

**Survival of probiotic lactic acid bacteria in the intestinal tract, their  
adhesion to epithelial cells and their ability to compete with  
pathogenic microorganisms**

**By**

**Marelize Botes**

Dissertation presented for the Degree of Doctor of Philosophy (Microbiology) at  
the University of Stellenbosch



Promoter: Prof. L.M.T. Dicks

March 2008

## **Declaration**

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and I have not previously, in its entire or in part, submitted it at any university for a degree.

Marelize Botes

Date:

## Summary

Research on probiotics has increased over the past years, which led to commercialization of a number of probiotic supplements and functional foods. *In vitro* assays such as tolerance to acid and bile, adhesion to mucus and epithelial cells, antimicrobial activity and antibiotic resistance tests are performed to screen lactic acid bacteria for probiotic properties.

*Enterococcus mundtii* ST4SA produces an antimicrobial peptide (peptide ST4SA) with activity against Gram-positive and Gram-negative bacteria. *Lactobacillus plantarum* 423 produces plantaricin 423, a typical class II bacteriocin, active against a number of Gram-positive bacteria.

A gastro-intestinal model (GIM) simulating the gastro-intestinal tract (GIT) of infants, was developed to study the survival of *E. mundtii* ST4SA and *L. plantarum* 423 and evaluate them as possible probiotics. Growth of the two strains in the GIM was compared to the growth of commercially available probiotics. Infant milk formulations were used as growth medium. Changes in pH, the addition of bile salt and pancreatic juice, and intestinal flow rates were controlled by peristaltic pumps linked to a computer with specifically designed software.

Strain ST4SA was sensitive to low pH and high concentrations of bile salts. Growth of strain ST4SA was repressed in the first part of the GIM, however, the cells recovered in the ileum. Strain 423 was also sensitive to acidic conditions. However, the cells withstood the presence of bile and pancreatin in the first part of the GIT. Neither of the two strains displayed bile salt hydrolase (BSH) activity. Both strains were resistant to amoxicillin, ampicillin, chloramphenicol, cefadroxil, roxithromycin, meloxicam, doxycycline, erythromycin, novobiocin, rifampicin, tetracyclin, bacitracin, ofloxacin and cephalosporin, anti-inflammatory drugs Na<sup>+</sup>-diklofenak and ibuprofen, and painkillers codeine terprim hydrate aminobenzoic acid, metamizole aspirin and paracetamol. Strain 423 was resistant to ciprofloxacin. Genes encoding cytolysin, non-cytolysin  $\beta$ -hemolysin and cell aggregation substances were detected on the genome of strain ST4SA but they were not expressed. *L. plantarum* 423 does not contain genes encoding gelatinase, cell aggregation, enterococcus surface protein, hemolysin, non-cytolysin  $\beta$ -hemolysin and enterococcus endocarditis antigen. Both strains inhibited the growth of *Listeria monocytogenes* ScottA in the GIM. Survival of the strains improved when used in combination and compared well with the survival of commercially available probiotics.

Adhesion to epithelial cells is an important prerequisite for bacterial colonization in the GIT. The adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 was studied using Caco-2 (human colon carcinoma epithelial) cells. Both strains revealed good adhesion compared to other probiotic strains. No correlation was found between hydrophobicity, auto-aggregation and adhesion to Caco-2 cells. Antibiotics and anti-inflammatory medicaments had a negative effect on adhesion. Different combinations of proteins were involved in the adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells. *E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA were stained with fluorescent dyes to visualize adhesion to Caco-2 cells. Adhesion of *L. monocytogenes* ScottA to Caco-2 cells was not reduced in the presence of strains ST4SA and 423. Cell-free culture supernatants of both strains inhibited the invasion of *L. monocytogenes* ScottA. The cell structure of Caco-2 cells changed in the presence of *L. monocytogenes* ScottA. Strains ST4SA and 423 protected Caco-2 cells from deforming.

## Opsomming

Navorsing op probiotika het die afgelope tyd drasties toegeneem en aanleiding gegee tot die kommersialisering van 'n groot hoeveelheid probiotiese supplemente en funksionele voedselsoorte. *In vitro* studies, soos bv. weerstand teen suur en gal, vashegting aan mukus en epiteelselle, antimikrobiële aktiwiteit en weerstand teen antibiotika word uitgevoer om te bepaal of melksuurbakteriëe aan probiotiese standaarde voldoen.

*Enterococcus mundtii* ST4SA produseer 'n peptied met antimikrobiële werking teen Gram-positiewe en Gram-negatiewe bakteriëe. *Lactobacillus plantarum* 423 produseer 'n tipiese klas II bakteriosien, plantarisien 423, met aktiwiteit teen sekere Gram-positiewe bakteriëe.

'n Gastro-intestinale model (GIM) wat die spysverteringskanaal (SVK) van babas simuleer, is ontwikkel om die oorlewing van *E. mundtii* ST4SA en *L. plantarum* 423 te bepaal en hul eienskappe met dié van kommersiële probiotiese stamme te vergelyk. Babamelk formules is as groeimedium gebruik. Verandering in pH, byvoeging van galsoute en pankreassappe, en intestinale vloei is met behulp van peristaltiese pompe gereguleer wat seine vanaf 'n spesiaal ontwikkelde rekenaarprogram ontvang.

*E. mundtii* ST4SA was sensitief vir lae pH en hoë galsoutkonsentrasies en groei is in die eerste deel van die GIM onderdruk. Selgetalle het wel in die ileum herstel. Stam 423 was ook sensitief vir lae pH, maar het die galsout- en pankreatienvlakke in die laer deel van die SVK weerstaan. Geen galsout-hidrolase aktiwiteit is by enige van die twee stamme gevind nie.

Beide stamme het weerstand getoon teen amoksillien, ampicillien, chloramfenikol, cefadroksiel, roksitromisien, meloksikam, doksisisiklien, eritromisien, novobiosien, rifampisien, tetrasiklien, basitrasien, oflaksasien, kefazolien, die anti-inflammatoriese medikamente Na<sup>+</sup>-diklofenak en ibuprofen, en die pynstillers kodeïnterprimhidraataminobensoësuur, metamisoolaspirien en parasetamol. *L. plantarum* 423 was bestand teen ciprofloksasien. Gene wat kodeer vir sitolisien, nie-sitolisien  $\beta$ -hemolisien III en sel-aggregasie is op die genoom van *E. mundtii* ST4SA gevind, maar word nie uitgedruk nie. *L. plantarum* 423 besit nie die gene wat vir gelatinase, sel-aggregasie substansies, enterokokkus selwandproteïen, hemolise, nie-sitolisien  $\beta$ -hemolisien en enterokokkus endokarditis antigeen kodeer nie. Albei stamme inhibeer die groei van *Listeria*

*monocytogenes* ScottA in die GIM. Die twee stamme in kombinasie het tot beter oorlewing in die GIM gelei. Stamme ST4SA en 423 vergelyk goed met kommersieël beskikbare probiotika.

Vashegting van probiotiese stamme aan epiteelselle is belangrik vir kolonisering in die SVK. Vashegting van *E. mundtii* ST4SA en *L. plantarum* 423 is bestudeer deur van Caco-2 (kolon epiteel) selle van die mens gebruik te maak. Die aanhegting van beide stamme aan Caco-2 selle het goed vergelyk met kommersieël beskikbare probiotiese stamme. Geen korrelasie is gevind tussen hidrofobisiteit, aggregasie en vashegting aan Caco-2 selle nie. Antibiotika en anti-inflammatoriese medikamente het 'n negatiewe effek op vashegting gehad. Verskillende kombinasies van proteïene is betrokke in die vashegting van *E. mundtii* ST4SA en *L. plantarum* 423 aan Caco-2 selle. *E. mundtii* ST4SA, *L. plantarum* 423 en *L. monocytogenes* ScottA is met fluoreserende kleurstowwe gemerk om vashegting aan Caco-2 selle te monitor. Vashegting van *L. monocytogenes* ScottA aan Caco-2 selle is nie deur die teenwoordigheid van stamme ST4SA en 423 beïnvloed nie. Sel-vrye kultuursupernatante van beide stamme het die binnedring van *L. monocytogenes* ScottA verhoed. Die selstruktuur van Caco-2 selle het in die teenwoordigheid van *L. monocytogenes* ScottA van vorm verander. *E. mundtii* ST4SA en *L. plantarum* 423 het die Caco-2 selle teen vervorming beskerm.

## **ACKNOWLEDGEMENTS**

My sincere thanks and gratitude for the following persons and institution:

My promoter, Prof. Dicks for his guidance, advice and positive criticism as well as constant support and enthusiasm.

My fellow colleagues in the lab for moral support and that you always made sure I laugh at least once a day. You are very special to me.

Ben Loos at the Department of Physiological Sciences for assistance with the fluorescent microscope, patience with me and interest in my project.

The National Research Foundation of South Africa for funding.

My parents for their motivation since the start of my academic career.

My husband, Cobus, who has supported me every day. Thank you for all your love, motivation and constant belief in my capabilities. I love you more every day.

This dissertation is dedicated to you.

## Contents

	<b>Page</b>
<b>CHAPTER 1</b>	
1. Introduction	2
References	5
<b>CHAPTER 2</b>	
<b>Probiotics: The battle ‘for life’: A review</b>	
1. Introduction	12
2. Digestion of food in the gastro-intestinal tract	13
3. Survival of micro-organisms in the gastro-intestinal tract	16
4. Adhesion	22
5. <i>In vitro</i> and <i>in vivo</i> fluorescent imaging	32
6. Characterization of antimicrobial agents produced by LAB	34
7. Safety of probiotic bacteria	41
8. Health benefits	51
9. Conclusions and future perspectives	73
References	73
<b>CHAPTER 3</b>	
<b>Evaluation of <i>Enterococcus mundtii</i> ST4SA and <i>Lactobacillus plantarum</i> 423 as probiotics and growth studies in a model simulating an infant gastro-intestinal tract</b>	
Abstract	115
1. Introduction	116
2. Materials and Methods	117
3. Results	121
4. Discussion	123
Acknowledgements	126
References	126



	<b>Page</b>
Tables and figures	133
 <b>CHAPTER 4</b>	
<b>Adhesion of <i>Enterococcus mundtii</i> ST4SA and <i>Lactobacillus plantarum</i> 423 to Caco-2 cells</b>	
1. Abstract	141
2. Introduction	141
3. Materials and Methods	142
4. Results	148
5. Discussion	150
Acknowledgements	153
References	153
Tables and figures	159
 <b>CHAPTER 5</b>	
1. General discussion	168
2. Concluding remarks and future perspectives	170
References	171

# CHAPTER 1

## **Introduction**

This chapter is written according to the style of International Journal of Food Microbiology.

## Introduction

Lactic acid bacteria (LAB) are generally regarded as safe (GRAS) and form part of the probiotic concept which received considerable attention over the past years (Salminen et al., 1998). The concept of eating or applying live bacteria for a health benefit goes back a hundred years (Metchnikoff, 1907). Probiotics are defined as “Living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition” (Gorbach, 2002) and play a role in the prevention or treatment of infectious diseases, irritable bowel syndrome, allergies, lactose intolerance, colon cancer and chronically high cholesterol levels (Andersson et al., 2001).

Criteria for selection of probiotic strains have only recently been formulated by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO). Some of the most important criteria are gastric and bile acid resistance, adhesion to mucus and/or human epithelial cells, competition with pathogens for adhesion sites, growth inhibition of potentially pathogenic bacteria, bile salt hydrolase activity and, in the case of vaginal applications, resistance to spermicides (FAO/WHO, 2002).

Several *in vitro* techniques are used to evaluate probiotic strains. Survival in the presence of acid, bile, enzymes and pancreatic juice is studied under conditions simulating the gastro-intestinal tract (Conway et al., 1987; Fernández et al., 2003; Lin et al., 2006; Pinto et al., 2006). These *in vitro* tests are time consuming and are not a true representation of conditions *in vivo*. *In vitro* models, simulating human gastro-intestinal conditions, were later developed. The SHIME, described by Molly and co-workers (1993), consisted of five reactors that represented the small and large intestine. Different *in vitro* gastro-intestinal models followed, e.g. an upper-gastro-intestinal model, a three-stage compound continuous culture system representing the proximal colon, and a model simulating the stomach and duodenum (Macfarlane et al., 1998; Mainville et al., 2005; Minekus et al., 1995). These models are based on a “fill and draw” system with reactors representing sections of the intestine. Each vessel is equipped with pH and temperature probes and entry ports for solutions such as medium, HCl, NaOH and oxbile (Mainville et al., 2005; Macfarlane et al., 1998; Minekus et al., 1995; Molly et al., 1993). The advantage of an *in vitro* model lies in the evaluation of all components in one system.

After surviving the harsh conditions in the stomach, probiotic bacteria are encountered by the mucus and epithelial cells in the small intestine. Physico-chemical properties of probiotic bacteria draw them closer to the mucus and they may come in close contact to adhesion sites on epithelial cells (Guimonde & Salminen, 2006). Adhesion is facilitated by cell-surface proteins (including S-layer proteins), carbohydrates, hemagglutinins and lipoteichoic acids (Adlerberth et al., 1996; Andreu et al., 1995; Bernet et al., 1994; Frece et al., 2005; Granato et al., 1999; Greene & Klaenhammer, 1994; Roos & Jonsson, 2002; Satoh et al., 1999; Vidal et al., 2002). Adhesion increases the retention time of probiotic cells to the intestine, which in turn prevents colonization of pathogens and modulates the immune system of the host (Guimonde & Salminen, 2006). Several *in vitro* epithelial cell models have been developed to study adhesion. Caco-2 cells, a human colonic adenocarcinoma cell line, differentiate spontaneously and grow as cylindrical polarized cells, with microvilli on the apical side and tight junctions between adjacent cells. These cells produce hydrolases similar to levels encountered in normal epithelial cells (Sambuy et al., 2005). Such *in vitro* models are useful to select probiotics with adhesion properties and to calculate predicted dose-responses in clinical trials (Tuomola & Salminen, 1998).

Fluorescent staining has recently been used to study bacterial adhesion (Fuller et al., 2000). Lectin binds specifically to *N*-acetylglucosamine in the peptidoglycan layer of Gram-positive bacteria. Cell-permeating nucleic acid stains, e.g. intercalating dyes such as ethidium bromide and propidium iodide and minor-groove binders such as DAPI and Hoechst dyes, stain Gram-negative bacteria (Mason et al., 1998). Fluorescent staining is more specific and is used to detect specific strains in a complex environment such as the GIT.

Lactobacilli compete with pathogens for adhesion sites, as observed for *Lactobacillus rhamnosus* GG and *Lactobacillus casei* Shirota (Lee et al., 2003). Competitive exclusion might also involve bacteriocins produced by LAB (Klaenhammer, 1988; Tagg et al., 1976). These peptides are antimicrobial and could be used in combination with other antibiotics (Ouweland & Vesterlund, 2004). Bacteriocins of LAB (including the enterocins) have been divided into three major classes i.e. lantibiotics, small heat-stable peptides and large heat-labile proteins. Another mechanism of probiotic bacteria to compete with pathogenic bacteria is co-aggregation (Reid et al., 1988). Co-aggregation delays the removal of probiotic bacteria from the intestinal epithelium and prevents colonization by pathogenic bacteria (Collado et al., 2007; Reid et al., 1988; Schachtsiek et al., 2004; Schellenberg et al., 2006).

The use of probiotics as health supplements and as natural alternatives to therapeutic antibiotics is increasing. However, a few cases of bacteremia caused by LAB have been reported (Cannon et al., 2005; Donohue and Salminen, 1996; Gasser, 1994; Husni et al., 1997; Vesterlund et al., 2007). Probiotic strains should be assessed for safety by conducting studies on their intrinsic properties and pharmacokinetics and interactions with the host (Salminen et al., 1998; Saxelin et al., 2005). Systemic infections, deleterious metabolic activity, excessive immune stimulation and risk of gene transfer are four examples of adverse side-effects (Salminen et al., 1998). Bacteria causing these side-effects are mostly characteristic for the host's own micro-flora and are not associated with lactobacilli and bifidobacteria in comparison to other bacteria (Salminen et al., 1998). The greatest risk for safety is the use of multi-drug resistant probiotics and the spread of antibiotic resistance genes to pathogenic bacteria. Lactic acid bacteria, like all other bacteria, exchange antibiotic resistance genes to enhance their own resistance (Courvalin, 2006). Enterococci are known to harbor virulence factors that may contribute to pathogenesis (Franz & Holzapfel, 2004). Examples of these are aggregation substances encoded by pheromone-responsive plasmids (observed for *E. faecalis*), sex pheromones,  $\beta$ -haemolysin/bacteriocin or cytotoxin, enterococcus surface proteins, enterococcus endocarditis antigen (observed for *E. faecalis* and *E. faecium*), gelatinase, extracellular metallo-endopeptidase, hyaluronidase and capsules (Chow et al., 1993; Clewell et al., 2000; Franz et al., 2003; Franz & Holzapfel, 2004; Gilmore et al., 1994; Huebner et al., 1999; Ike et al., 1984; Jett et al., 1992; 1994; Su et al., 1991; Toledo-Arana et al., 2001).

*Enterococcus mundtii* ST4SA isolated from soybeans, produces an antimicrobial peptide active against various Gram-positive and Gram-negative bacteria. *Lactobacillus plantarum* 423, isolated from sorghum beer, produces a typical class II bacteriocin active against Gram-positive bacteria. Preliminary studies have indicated that both strains have probiotic properties. In this study the probiotic properties of *E. mundtii* ST4SA and *L. plantarum* 423 are evaluated and compared to those of commercially available probiotics.

The following objectives were formulated:

#### Survival properties

- To develop a computerized model simulating the gastro-intestinal tract of infants.
- To determine the survival of *E. mundtii* ST4SA and *L. plantarum* 423 in the gastro-intestinal model (GIM) and compare the strains to commercially available probiotics.

- To assess the safety of strains ST4SA and 423 in terms of antibiotic resistance and presence of virulence factors.

#### Adhesion properties

- To determine the hydrophobicity, auto- and co-aggregation properties of strains ST4SA and 423.
- To determine the adhesion of strains ST4SA and 423 to Caco-2 cells and compare them to commercially available probiotic strains.
- To determine if *E. mundtii* ST4SA and *L. plantarum* 423 are able to compete with other cells for binding to epithelial cells.
- To determine the effect of bile, pancreatin and antibiotics on the adhesion of strains ST4SA and 423 to epithelial cells.
- Visualization of *E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA adhesion to Caco-2 cells by fluorescent staining.

#### References

- Adlerberth, I., Ahrné, S., Johansson, M.-L., Molin, G., Hanson, L.A., Wold, A.E., 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Applied and Environmental Microbiology* 62, 2244-2251.
- Andersson, H., Asp, N.-G., Bruce, A., Roos, A., Wadström, T., Wold, A.E., 2001. Health effects of probiotics and prebiotics. A literature review on human studies. *Scandinavian Journal of Nutrition* 45, 58-75.
- Andreu, A., Stapleton, A.E., Fennell, C.L., Hillier, S.L., W. E. Stamm, W.E., 1995. Hemagglutination, adherence and surface properties of vaginal *Lactobacillus* species. *Journal of Infectious Diseases* 171, 1237–1243.
- Bernet, M.F., Brassart, D., Neeser, J.R., Servin, A.L., 1994. *Lactobacillus acidophilus* La1 binds to cultured human intestinal cell lines and inhibits cell-attachment and cell invasion by enterovirulent bacteria. *Gut* 35, 483-489.
- Cannon, J.P., Lee, T.A., Bolanos, J.T., Danziger, L.H., 2005. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. *European Journal of Clinical Microbiology* 24, 31-40.

- Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., Zervos, M.J., 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in-experimental enterococcal endocarditis. *Antimicrobial Agents in Chemotherapy* 37, 2474-2477.
- Clewell, D.B., An, F.Y., Flannagan, S.E., Antiporta, M., Dunny, G.M., 2000. Enterococcal sex pheromone precursors are part of signal sequences for surface lipoproteins. *Molecular Microbiology* 35, 246-247.
- Collado, M.C., Meriluoto, J., Salminen, S., 2007. Interactions between pathogens and lactic acid bacteria: aggregation and co-aggregation abilities. *European Journal of Food Research Technology*, in press.
- Conway, P.L., Gorbach, S.L., Goldin, B.R., 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cell. *Journal of Dairy Science* 70, 1-12.
- Courvalin, P., 2006. Antibiotic resistance: The pros and cons of probiotics. *Digestive and Liver Disease* 38, (suppl.2), S261-265.
- Donohue, D.C., Salminen, S., 1996. Safety assessment of probiotic bacteria. *Asia Pacific Journal of Clinical Nutrition* 5, 25–28.
- FAO/WHO, 2002. Guidelines for the Evaluation of Probiotics in Food. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food (<ftp://ftp.fao.org/es/esn/food/wgreport2.pdf>).
- Fernández, M.F., Boris, S., Barbés, C., 2003. Probiotic properties of human lactobacilli strains to be used in the gastro-intestinal tract. *Journal of Applied Microbiology* 94, 449-455.
- Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H., Holzapfel, W.H., 2003. Enterococci in foods-a conundrum for food safety. *International Journal of Food Microbiology* 88, 105-122.
- Franz, C.M.A.P., Holzapfel, W.H., 2004. The genus *Enterococcus*: Biotechnological and safety issues. In: Salminen, S., von Wright, A., Ouwehand, A., (Eds.), *Lactic acid bacteria Microbiological and Functional aspects*, 3<sup>rd</sup> ed., Revised and expanded, Marcel Dekker, inc.
- Frece, J., Kos, B., Svetec, I.K., Zgaga, Z., Mrša, V., Šušková, J., 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology* 98, 285-292.
- Fuller, M.E., Streger, S.H., Rothmel, R.K., Mailloux, B.J., Hall, J.A., Onstott, T.C., Fredrickson, J.K., Balkwill, D.L., DeFlaun, J.A., 2000. Development of a vital fluorescent staining

- method for monitoring bacterial transport in subsurface environments. *Applied and Environmental Microbiology* 66, 4486-4496.
- Gasser, F., 1994. Safety of lactic acid bacteria and their occurrence in human clinical infections. *Bulletin De L'Institute Pasteur* 92, 45-67.
- Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R., Clewell, D.B., 1994. Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *Journal of Bacteriology* 176, 7335-7344.
- Gorbach, S.I., 2002. Probiotics in the Third Millennium. *Digestive and Liver Disease* 34, (Suppl.2), 2-7.
- Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., Brassart, D., 1999. Cell-surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Applied and Environmental Microbiology* 65, 1071-1077.
- Greene, J.D., Klaenhammer, T.R., 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. *Applied and Environmental Microbiology* 60, 4487-4494.
- Gueimonde, M., Salminen, S., 2006. New methods for selecting and evaluating probiotics. *Digestive and Liver Disease* 38 (Suppl. 2), S242-S247.
- Huebner, J., Wang, Y., Krueger, W.A., Madoff, L.C., Martirosian, G., Biosot, S., Goldmann, D.A., Kasper, D.L., Tzianabos, A.O., Pier, G.B., 1999. Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*. *Infection and Immunity* 67, 1213-1219.
- Husni, R.N., Gordon, S.M., Washington, J.A., Longworth, D.L., 1997. *Lactobacillus* bacteremia and endocarditis: review of 45 cases. *Clinical Infectious Diseases* 25, 1048-1055.
- Ike, Y., Hashimoto, H., Clewell, D.B., 1984. Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. *Infection and Immunity* 45, 528-530.
- Jett, B.D., Jensen, H.G., Nordquist, R.E., Gilmore, M.S., 1992. Contribution of the pAD1-encoded cytolytic toxin to the severity of experimental *Enterococcus faecalis* endophthalmitis. *Infection and Immunity* 60, 2445-2452.
- Jett, B.D., Huycke, M.M., Gilmore, M.S., 1994. Virulence of enterococci. *Clinical Microbiology Reviews* 7, 462-478.
- Klaenhammer, T.R., 1988. Bacteriocins of lactic acid bacteria. *Biochimie* 70, 337-349.



- Lee, Y.K., Lin, C.Y., Teng, W.L., Ouwehand, A.C., Tuomola, E., Salminen, S., 2000. Qualitative approach in the study of adhesion of lactic acid bacteria on intestinal cells and their competition with enterobacteria. *Applied and Environmental Microbiology* 66, 3692-3697.
- Lee, Y.-K., Puong, K.-Y., Ouwehand, A.C. and Salminen, S., 2003. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *Journal of Medical Microbiology* 52, 925-930.
- Lin, W.-H., Hwang, C.-F., Chen, L.-W., Tsen, H.-Y., 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. *Food Microbiology* 23, 74-81.
- Macfarlane, G.T., Marfarlane, S., Gibson, G.R., 1998. Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial Ecology* 35, 180-187.
- Mainville, I., Arcand, Y., Farnworth, E.R., 2005. A dynamic model that simulates the human upper gastro-intestinal tract for the study of probiotics. *International Journal of Food Microbiology* 99, 287-296.
- Mason, D.J., Shanmuganathan, S., Mortimer, F.C., Gant, V.A., 1998. A fluorescent Gram-stain for flow cytometry and epifluorescence microscopy. *Applied and Environmental Microbiology* 64, 7, 2681-2685.
- Metchnikoff, E., 1907. *The prolongation of life*, London: William Heinemann.
- Minekus, M., Marteau, P., Havenaar, R., Huis in't Veld, J.H.J., 1995. A multicompartmental dynamic computer-controlled model simulating the stomach and the small intestine. *Alternatives to Laboratory Animals* 23, 197-209.
- Molly, K. Van de Woestyne, M., Verstraete, W., 1993. Development of a 5-step multi-chamber reator as a simulation of the human intestinal microbial ecosystem. *Applied and Microbiological Biotechnology* 39, 254-258.
- Ouwehand, A.C., Vesterlund, S., 2004. Antimicrobial components from lactic acid bacteria. In: Salminen, S., von Wright, Ouwehand, A., (Eds.), *Lactic acid bacteria, Microbiological and Functional aspects*, Marcel Dekker, Inc., pp. 375-395.
- Pinto, M.G.V., Franz, M.A.P., Schillinger, U., Holzapfel, W.H. 2006. *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. *International Journal of Food Microbiology* 109, 205-214.

- Reid, G., McGoarty, J.A., Angotti, R., Cook, R.L., 1988. *Lactobacillus* inhibitor production against *Escherichia coli* and co-aggregation ability with uropathogens. Canadian Journal of Microbiology 34, 344.
- Roos, S., Jonsson, H., 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology 148, 433-442.
- Salminen, S. Bouley, C., Boutron-Ruault, M.C., 1998. Functional food science and gastrointestinal physiology and function. British Journal of Nutrition 80, (suppl.1), 147–171.
- Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stamatii, A., Zucco, F., 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biology and Toxicology 21, 1-26.
- Satoh, E., Leer, R.J., Conway, P.L., Pouwels, P.H., 1999. Mucus adhesion promoting protein of *Lactobacillus reuteri* 104R: 6<sup>th</sup> Symposium on Lactic acid Bacteria, Veldhoven, the Netherlands, 9.
- Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., De Vos, W.M., 2005. Probiotic and functional microbes: from markets to mechanisms. Current Opinion in Biotechnology 16, 204-211.
- Schachtsiek, M., Hammes, W.P., Hertel, C., 2004. Characterization of *Lactobacillus coryniformis* DSM 20001T surface protein Cpf mediating co-aggregation with and aggregation among pathogens. Applied and Environmental Microbiology 70, 7078-7085.
- Schellenberg, J., Smoragiewicz, W., Karska-Wysocki, B., 2006. A rapid method combining immunofluorescence and flow cytometry for improved understanding of competitive interactions between lactic acid bacteria (LAB) and methicillin resistant *S. aureus* (MRSA) in mixed culture. Journal of Microbiology Methods 65, 1-9.
- Su, Y.A., Sulavik, M.C., He, P., Mäkinen, P., Fiedler, S., Wirth, R., Clewell, D.B., 1991. Nucleotide sequence of the gelatinase gen (gelE) from *Enterococcus faecalis* subsp. *liquefaciens*. Infection and Immunity 59, 415-420.
- Tagg, J.R., Dajani, A.S. Wannamaker, L.W., 1976. Bacteriocins of Gram-positive bacteria. Microbiology Reviews 40, 722–756.
- Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.L., Cacarella, C., Lamata, M., Amorenta, B., Leiva, J., Penandés, J.R., Lasa, I., 2001. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. Applied and Environmental Microbiology 67, 4538-4545.

- Tuomola, E.M. (née Lehto), Salminen, S.J., 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International Journal of Food Microbiology* 41, 45-51.
- Vesterlund, S., Paltta, J., Karp, M., Ouwehand, A.C., 2005. Adhesion of bacteria to resected human colonic tissue: Quantitative analysis of bacterial adhesion and viability. *Research in Microbiology* 156, 238-244.
- Vesterlund, S., Vankerckhoven, V., Saxelin, M., Goossens, H., Salminen, S., Ouwehand, A.C., 2007. Safety assessment of *Lactobacillus* strains: Presence of putative risk factors in faecal, blood and probiotic isolates. *International Journal Food Microbiology* 116, 325-331.
- Vidal, K.A., Donnet-Hughes, Granato, D., 2002. Lipoteichoic acids from *Lactobacillus johnsonii* strain La1 and *Lactobacillus acidophilus* strain La10 antagonize the responsiveness of human intestinal epithelial HT29 cells to lipopolysaccharide and gram-negative bacteria. *Infection and Immunity* 70, 2057-2064.

# CHAPTER 2

## **Probiotics: The battle ‘for life’: A review**

Prepared for publication in International Journal of Food Microbiology

## **Probiotics: The battle ‘for life’: A review**

**M. Botes, K. ten Doeschate, L.M.T. Dicks**

*Department of Microbiology, University of Stellenbosch, Stellenbosch, 7600, South Africa*

### **1. Introduction**

A hundred years have passed since Metchnikoff described the health benefits of milk fermented with specific strains (Metchnikoff, 1907). Since then, many lactic acid bacteria with probiotic properties have been isolated from plants, animals and fermented foods. Many claims relating to probiotic properties have been made, which varies from the prevention of infectious diseases (Rolfe, 2000), curing of irritable bowel syndrome, alleviation of allergies, digestion of lactose and lowering of serum cholesterol levels (Andersson et al., 2001) to prevention of cancer (Gibson & Macfarlane, 1994; Morotomi et al., 1990). It is thus not surprising that over the years many definitions for probiotics have been proposed. Marteau et al. (2002) defined probiotics as “microbial cell preparations or components of microbial cells that have a beneficial effect on health and well-being”. Gorbach (2002) defined probiotics as “living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition”. Recent definitions tend to focus more on specific effects, in particular stimulation of the immune system by well-defined strains (Isolauri et al., 2004). This posed the question whether any given micro-organism that adheres to any one or more of these definitions is considered a probiotic (Fioramonti et al., 2003). To fuel the debate further, a number of recently published papers have suggested that probiotics may cause side-effects such as systemic infections, deleterious metabolic activities and excessive immune stimulation in susceptible individuals. Reports on virulence and the possible exchange of genes encoding antibiotic resistance (Marteau, 2002) have emphasized the importance of having the correct safety precautions in place when selecting a probiotic.

Regulatory guidelines were implemented in 2002 (FAO/WHO) to ensure safe and reliable products. A probiotic should be (a) resistant to gastric and bile acid, (b) adhere to mucus and/or human epithelial cells, (c) inhibit the growth and colonization of pathogens, and if used intravaginally, (d) be resistant to spermicides (FAO/WHO, 2002). Most of these tests are performed *in vitro* (Conway et al., 1987; Floch, 2002; Lin et al., 2006). Studies with human cell lines such as Caco-2, HT-29 and HT29-MTX provide an opportunity to study adhesion to mucus,

glycoproteins and epithelial cells (Gopal et al., 2001; Kirjavainen et al., 1998; Ouwehand et al., 1999; Sambuy et al., 2005). Adhesion to epithelial cells differ with the use of different cell lines, e.g. bacterial strains have a higher affinity to HT29-MTX cells than Caco-2 cells (Shillinger et al., 2005). Other factors of importance are cell-surface proteins (Adlerberth et al., 1996; Bernet et al., 1994; Roos & Jonsson, 2002; Satoh et al., 1999), carbohydrates (Granato et al., 1999; Vidal et al., 2002), hemagglutinins (Andreu et al., 1995), S-layer proteins (Frece et al., 2005) and lipoteichoic acids (Greene and Klaenhammer, 1994). *In vitro* studies on cell lines are important in that they bridge the gap between “test-tube” research and animal or human studies. However, *in vivo* trials have to be conducted in final evaluations (Charteris et al., 1998; Jacobsen et al., 1999; Mishra & Prasad, 2005).

Genomic analyses have proved valuable in probiotic studies. Examples are the F1F0-ATPase system, encoded by the *atp* operon, ornithine decarboxylase (La996) and the role it plays in acid tolerance of *Lactobacillus acidophilus* NCFM (Alterman et al., 2005), bile salt hydrolase (BSH) and bile transport by *Lactobacillus johnsonii* NCC 533 (Pridmore et al., 2004). Bron and co-workers (2004) described fourteen genes and gene clusters encoding cytoplasmic membrane and cell-wall-associated functions involved in bile tolerance of *Lactobacillus plantarum* WCFS1.

This review focuses on the properties of probiotic lactic acid bacteria, the gastro-intestinal tract, mode of action, safety of probiotics, and methods used to study these organisms.

## **2. Digestion of food in the gastro-intestinal tract**

Food enters the stomach and forms concentric circles in the body and fundus so that the latest food is closest to the esophagus and the oldest food nearest the wall of the body of the stomach. Digestive juices, secreted from glands on the surface of the stomach wall (Table 1), come into contact with stored food and the mucosa. Contractions towards the antrum blend secretions with the food, resulting in a murky, milky semi- fluid or paste known as chime. Peristaltic constrictor rings force the stomach contents towards the pylorus and into the duodenum. Lysozyme in the saliva weakens the cell wall of bacteria and facilitates the entering of thiocyanate ions into cells (Guyton, 1991).

The surface of the stomach is lined with mucus-secreting cells. The mucus, composed of water, electrolytes and several glycoproteins, acts as a lubricant that spreads food and other particles

across the intestinal surface. Mucus is not easily digested by gastro-intestinal enzymes and serves as a buffer against acids and alkalis. Oxyntic glands in the mucosa secrete hydrochloric acid, pepsinogen, intrinsic factor and mucus. Pyloric glands, also positioned in the mucosa, secrete mucus, pepsinogen and the hormone gastrin, which in turn regulates antral peristalsis and thus emptying of the stomach. Feedback signals from the duodenum, i.e. enterogastric feedback reflexes and hormonal feedback, help to force the chyme through the small intestine at 0.5 to 2 cm/sec. Chyme takes three to five hours to pass from the pylorus valve to the ileocecal valve (Guyton, 1991).

Table 1

Volumes and pH values of intestinal juices secreted daily (obtained from Guyton, 1991)

	Daily volume (ml)	pH
Saliva	1000	6.0-7.0
Gastric secretion	1500	1.0-3.5
Pancreatic secretion	1000	8.0-8.3
Bile	1000	7.8
Small intestine secretion	1800	7.5-8.0
Brunner's gland secretion	200	8.0-8.9
Large intestine secretion	200	7.5-8.0
Total	6700	

Pancreatic juice composed of digestive enzymes and sodium bicarbonate is secreted in response to the presence of chyme in the upper sections of the small intestine. Bicarbonate levels increase to 145 milli equivalents of solute per liter (mEq/liter) and provide more alkali to neutralize the acid chyme. The presence of chyme in the small intestine stimulates secretion of cholecystokinin and secretin. Cholecystokinin stimulates contractions of the gallbladder and common bile duct and delivers bile to the duodenum. Secretin stimulates biliary duct cells to secrete bicarbonate and water. This expands the volume of bile and increases its flow to the intestine (Guyton, 1991). Bile is secreted by the liver, stored in the gallbladder and released in the duodenum (Taranto et al., 2006). The combination of bile salts, bilirubin, cholesterol, lecithin and electrolytes (Table 2) renders bile amphipathic and bactericidal (Guyton, 1991; Hofmann, 1994).

Table 2

Composition of bile (obtained from Guyton, 1991)

	Liver bile	Gallbladder bile
Water	97.5 g/dl	92 g/dl
Bile salts	1.1 g/dl	6 g/dl
Bilirubin	0.04 g/dl	0.3 g/dl
Cholesterol	0.10 g/dl	0.3-0.9 g/dl
Fatty acids	0.12 g/dl	0.3-1.2 g/dl
Lecithin	0.04 g/dl	0.3 g/dl
Na <sup>+</sup>	145 mEq/liter	130 mEq/liter
K <sup>+</sup>	5 mEq/liter	12 mEq/liter
Ca <sup>++</sup>	5 mEq/liter	23 mEq/liter
Cl <sup>-</sup>	100 mEq/liter	25 mEq/liter
HCO <sub>3</sub> <sup>-</sup>	28 mEq/liter	10 mEq/liter

Absorption of nutrients does not take place in the stomach, as it lacks the typical villous-type membrane. The intestinal mucosa has many folds (folds of Kerckring) which increase the surface area of the mucosa by approximately three-fold. These folds extend circularly along most of the intestine and are well developed in the duodenum and jejunum. Small villi that project approximately 1 millimeter from the surface of the mucosa cover the entire surface of the small intestine (Fig. 1). The villi are situated adjacent to each other in the upper small intestine and are almost completely in contact with each other. Villi in the distal small intestine are less compact. Each epithelial cell is characterized by brush border, consisting of about 600 microvilli, 1 micrometer in length and 0.1 micrometer in diameter, protruding into the intestinal chime. The folds of Kerckring, villi and microvilli increase the absorptive area of the mucosa up to 600-fold.

Once in the colon, the chime migrates with the help of sluggish contractions. Water and electrolytes are absorbed from the chime in the proximal half of the colon and fecal material is stored in the distal half. The mucosa in the large intestine is similar to the mucosa in the small intestine, but does not have villi. Epithelial cells of the large intestine secrete very few enzymes and are almost entirely covered by mucous cells. Large numbers of mucous cells are dispersed among other epithelial cells. Mucus contains high concentrations of bicarbonate ions to protect the intestinal wall from harmful faecal bacteria (Guyton, 1991). Protection is important, since more than  $10^{11}$  bacteria per gram dry matter has been reported in the colon (Guyton, 1991). Several hundred bacterial species have been isolated from faeces (Guyton, 1991). Approximately 40 of these species are grouped into six genera and account for 99 %



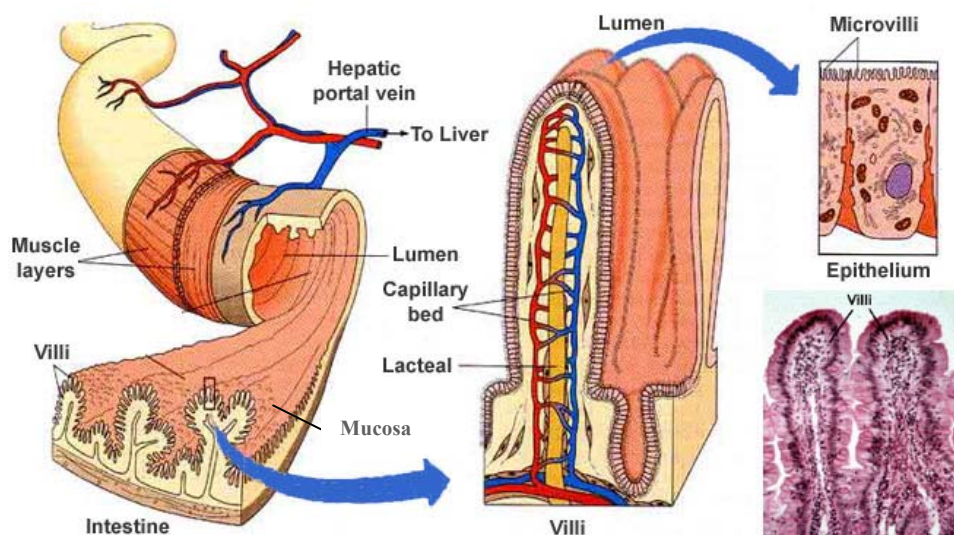


Fig. 1. A longitudinal section of the small intestine, showing the surface of mucosa covered by villi ([www.colorado.edu](http://www.colorado.edu)).

of the faecal biomass. Asporogenous anaerobes such as *Bacteroides*, *Bifidobacterium* and *Eubacterium* spp. predominate the colon. The micro-aerophilic species include streptococci, enterobacteria and lactobacilli. Facultative anaerobic cocci are represented by *Peptostreptococcus* spp., *Ruminococcus* spp., *Megasphaera elsdenii*, *Sarcina ventriculi*, *Enterococcus faecalis*, *Streptococcus bovis*, *Streptococcus equines* and *Streptococcus salivarius*. *Veillonella* and *Acidaminococcus* spp. present the Gram-negative anaerobic cocci (Cummings, 1997).

### 3. Survival of microorganisms in the gastro-intestinal tract

#### 3.1 Acid and bile tolerance

Physiological stress of bacteria begins in the stomach, where the pH may reach 1.5 (Lankaputhra and Shah, 1995). Bile secreted in the small intestine reduces the survival of bacteria by changing the composition of lipids and fatty acids in their cell membranes. These modifications may affect interactions between the membrane and the environment, thus permeability (Gilliland et al., 1977). Acid and bile tolerance are therefore considered key criteria in the selection of a probiotic (Lankaputhra and Shah, 1995). *In vitro* methods used to simulate gastro-intestinal conditions and select probiotic strains are listed in Table 3.

Table 3

*In vitro* methods used to evaluate potential probiotic strains.

**Simulation of Stomach acid:**

Growth medium or sterilized phosphate-buffered saline (PBS, 6.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.14 M NaCl, 3.0 mM KCl, pH 7.3) with pH values adjusted between 2.0 and 3.2 (Conway et al., 1987; Lin et al., 2006).

Electrolyte solution (6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l CaCl<sub>2</sub> and 1.2 g/l NaHCO<sub>3</sub>), adjusted to pH 2.5 (Pinto et al., 2006).

Gastric juice withdrawn from humans (Del Piano et al., 2006).

**Gastro-intestinal enzymes:**

Lysozyme (100ppm) and pepsin (0.3% w/v) added to acidic buffers (Pinto et al., 2006). Strains are incubated for 1 h to 3 h in acidic buffers and plated onto specific growth media.

**Bile acids:**

Different preparations of porcine and bovine bile are used, based on the presence of conjugated and unconjugated bile salts (Floch, 2002).

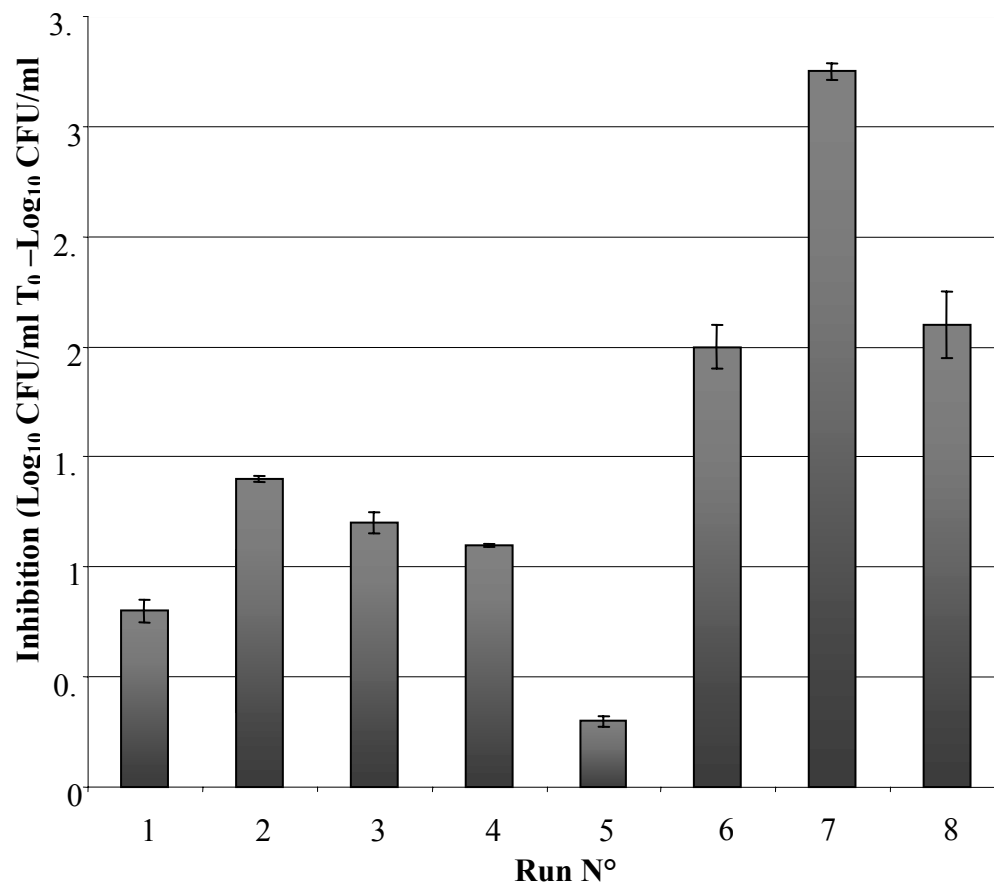
**Duodenum secretions:**

Artificial duodenum secretion (pH 7.2): 6.4 g/l NaHCO<sub>3</sub>, 0.239 g/l KCl, 1.28 g/l NaCl, 5 g/l bile salts and 1 g/l pancreatin (Fernández et al., 2003; Pinto et al., 2006; Zárate et al., 2000).

Cells are collected after exposure to the acidic buffers, incubated in 3 g/l (w/v) bile for 36 h and plated onto specific growth media (Gilliland & Walker, 1990; Lin et al., 2006; Yu & Tsen, 1993).

*In vitro* studies with human and simulated gastric juice revealed between 15 % and 45 % survival of microbial cells (strains of *L. plantarum*) in human gastric juice and 20 % survival of the same strains in simulated gastric juice (Del Piano et al., 2006). Viability is strain dependant and influenced by pH and exposure time. Food intake also protects bacteria during gastric passage (Del Piano et al., 2006). Milk has a protective effect on bacteria and the presence of pepsin and lysozyme increases inhibition (Fig. 2; Pinto et al., 2006). Bacterial strains isolated from the human gut revealed higher levels of acid resistance in simulated gastric juice (Conway, 1987). Strains with good colonizing properties were less resistant to low pH in *in vitro* studies (Charteris et al., 1998; Jacobsen et al., 1999; Mishra & Prasad, 2005). Strains of *Lactobacillus paracasei* showed low resistance to gastric acid *in vitro*, although survival in the gastro-intestinal tract was excellent (Charteris et al., 1998; Mishra & Prasad, 2005). Although *in vitro* assays may provide

information on acid tolerance, the method should be refined to correlate with *in vivo* tests, or validated by human clinical trials.



Run	1	2	3	4	5	6	7	8
Milk	+	+	+	-	+	-	-	-
Medium	MRS	Electrolyte	Electrolyte	Electrolyte	MRS	Electrolyte	MRS	MRS
Composition		solution	solution	solution		solution		
Lysozyme	-	-	+	+	+	-	+	-
pH	3	2.5	2.5	3	3	3	2.5	2.5
Pepsin	+	-	+	-	-	+	+	-

Fig. 2. Effect of different variables (presence of milk, lysosyme and pepsin, medium composition and pH) on *L. johnsonii* LA1 after 1 h in the presence of artificial gastric juice determined in eight experiments (Pinto et al., 2006). One milliliter samples were taken at the beginning of the each assay (T0) and 1 h later (T60). The response was calculated as the difference between the log units of the initial and final counts (inhibition: log CFU/mlT0 - log CFU/mlT60). The symbols + and - refer respectively to the presence and absence of milk, lysozyme and pepsin.

Multiple factors such as stress response systems and transcriptional regulators, elements involved in the maintenance of the cell envelope, energy metabolism, amino acid transport (putative role in pH homogenesis) and fatty acid or isoprenoid biosynthesis play a role in survival of bacteria in bile-rich environments (Begley et al., 2000; Breton et al., 2002). Cholic acid and deoxycholic acid (DCA) have a stronger inhibitory effect on intestinal aerobic and anaerobic bacteria compared to conjugated bile acids (Floch et al., 1972). The first evidence of DCA toxicity in lactic acid bacteria was reported by Taranto et al. (2006). DCA is more hydrophobic and toxic than taurodeoxycholate (TCDA). Electron microscopy showed severe distortion of the cell envelope, complete permeabilization of cells and prevention of glucose uptake by *Lactobacillus reuteri* CRL 1098. The lipid composition of the membrane, including the ratio of phospholipids to glycolipids, changed significantly. This can be explained by the improved physico-chemical properties of the membrane after a rapid adaptive response to bile by viable cells. However, sugar transport and permeability assays revealed changes in the integrity of the cell wall, leading to death (Taranto et al., 2006). *Lactobacillus* spp. differ significantly in bile tolerance and are less sensitive to human bile than bovine bile (Fig. 3; Del Piano et al., 2006).

Bile salt hydrolase (BSH) activity is a controversial subject. The phenomenon was initially associated with natural tolerance to bile salts (De Smet et al., 1994; Moser and Savage, 2001). However, recent studies have shown that bile salt resistance of lactobacilli could not be associated with the presence of BSH (Gilliland and Speck, 1977; Moser and Savage, 2001; Schmidt et al., 2001).

### *3.2 Mechanisms of survival in the gastro-intestinal tract*

The reaction of bacteria to intestinal conditions is not fully understood (De Vriese et al., 2006). Genome sequencing could be a new resolution and therefore the complete genome sequences of probiotic bacteria such as *L. plantarum* WCFS1, *L. acidophilus* NCFM and *L. johnsonii* NCC 533 were determined (Alterman et al., 2005; Kleerebezem et al., 2003; Pridmore et al., 2004). Genomic analysis together with studies done on their behavior in the gastro-intestinal tract could give more insight into the mechanisms behind probiotic functions (De Vos et al., 2004).

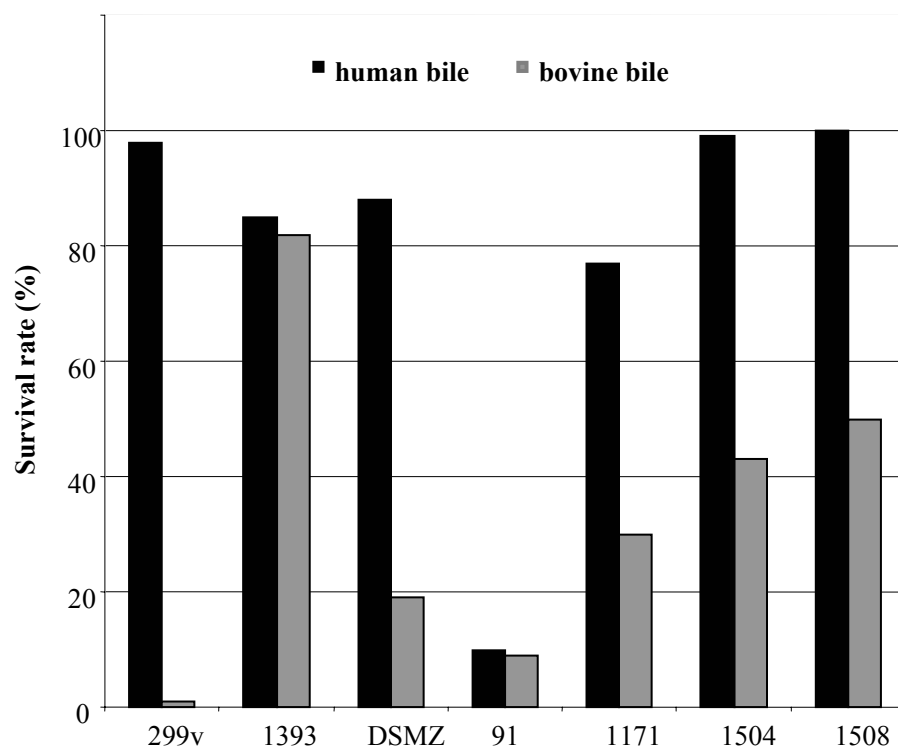


Fig. 3. Gastro-intestinal resistance of different strains of *L. plantarum* to 0.3% human bile and bovine bile salts (Del Piano et al., 2006).

Mechanisms used by Gram-positive bacteria in resistance to acid include proton pumps, amino acid decarboxylation, electrogenic transport systems, chaperones involved in repair/degradation of damaged proteins, incremental expression of regulators promoting local or global responses and changes in the structure of the cell envelope (Cotter & Hill, 2003). The  $F_1F_0$ -system encoded by the *atp* operon and ornithine decarboxylase (La996) was studied for *L. acidophilus* NCFM (Azcarate-Peril et al., 2004). A thioredoxin system and genes encoding glutathione reductase, NADH-oxidase and NADH-peroxidase were also identified (Altermann et al., 2005). *L. johnsonii* NCC 533 has three genes encoding BSH, one less than *L. plantarum* WCFS1 (Pridmore et al., 2004). The large number of BSH-encoding genes emphasizes the importance of this characteristic to survival of strains in the gastro-intestinal tract (Dambekodi & Gilliland, 1998; Pridmore et al., 2004).

Genes that are switched on under conditions simulating the gastro-intestinal tract were detected in several *in vitro* studies. Fragments of the *L. plantarum* WCFS1 genome were cloned upstream of a promoterless alanine racemase (*alr*) gene of *Lactococcus lactis* in a low copy number plasmid.

The plasmid library, which covered 98 % of the genome, was introduced into a *alr* deletion mutant (*L. plantarum* $\Delta$ *alr*) (Bron, Meijer et al., 2004). Clones that could complement the D-alanine auxotroph phenotype in the presence of 0.8 M NaCl were screened. Significantly higher production of *alr* in eight clones was detected that contained *L. plantarum* promoters preceding genes coding for different functions. These functions included an integral membrane protein, glycerate kinase, permease, short chain dehydrogenase and different hypothetical proteins. A specific regulation of genes was indicated by four promoters with the same conserved motive, not present on the chromosome (Bron, Meijer et al., 2004). Thirty-one genes, including 11 membrane- and cell wall-associated functions, five functions involved in redox reactions and five regulatory factors were induced in the presence of 0.1 % porcine bile (Bron, Molenaar et al., 2004). Growth of *L. plantarum* WCF1 on MRS agar, with or without 0.1% porcine bile, was compared by using DNA micro-arrays. Stress proteins, cell-envelope located proteins and proteins involved in redox reactions were up-regulated (Bron, Hoffer et al., 2004; Bron, Molenaar et al., 2004). These studies showed alterations in the cell wall that could protect the cell from harsh conditions (Bron, Hoffer et al., 2004). Genes involved in redox reactions which are up-regulated might be explained by different metabolic reactions under intestinal conditions.

A resolvase-based *in vivo* expression technology (R-IVET) was used to switch on the genes of *L. plantarum* WCFS1 in the intestinal tract of mice (Bron, Grangette et al., 2004). The genes coded for sugar-related functions, acquisition and synthesis of amino acids, nucleotides, cofactors, vitamins and stress-related functions (Fig. 4). Deletion of these genes resulted in reduced survival of *L. plantarum* WCFS1 in the gastro-intestinal tract. This indicated that *L. plantarum* WCFS1 adapts to different environmental conditions and that the series of functions are concentrated in a defined genomic region (Kleerebezem et al., 2003).

DNA micro-arrays were used to determine gene expression of *L. plantarum* 299v in surgically removed intestinal segments of potential colon cancer patients (De Vriese et al., 2006). The patients were fed a fermented oatmeal drink with *L. plantarum* 299v. Genes encoding sugar uptake and metabolism, amino acid biosynthesis, cell division and stress were up regulated. This indicated survival, metabolic activity and growth of *L. plantarum* attached to the human intestine (De Vriese et al., 2006). DNA micro-arrays combined with clinical studies may provide insight and new perspectives on *in vivo* host-microbe interactions (De Vriese et al., 2006).

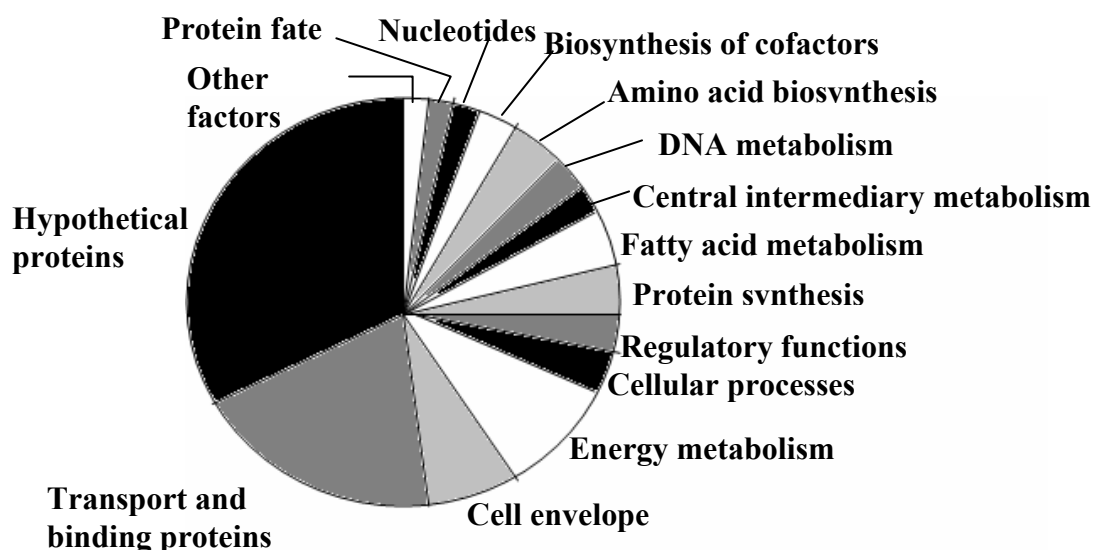


Fig. 4. Functional classes of genes of *L. plantarum* induced in the intestinal tract of rats, determined by using resolve-based *in vivo* expression technology (R-IVET) (Bron, Grangette et al., 2004).

## 4. Adhesion

### 4.1 Adhesion to mucus

Ingested micro-organisms are exposed to mucus in the small intestine after surviving through the stomach. Adhesion to mucus prevents flushing out of cells, especially in the small intestine with relatively high flow rates (Kirjavainen et al., 1998; Sanford, 1992). The *in vitro* models that are used to study adhesion of probiotic bacteria to mucus are listed in Table 4.

Kirjavainen and co-workers (1998) were the first to study the interaction between probiotic bacteria and glycoproteins. They found that adhesion of LAB to human intestinal tissue and mucus is strain dependent and reported the best adhesion with *Lactobacillus rhamnosus* GG (LGG). In a similar study, Tuomola et al. (1999) have shown that adhesion is concentration-dependent of adhesion sites. In another study, strains adhered stronger to the mucus of adults compared to the mucus of newborns (Kirjavainen et al., 1998). Less significant differences in the bacterial adhesion were recorded among infants of different ages. A positive correlation between increasing age and increased degradation of mucin exists between birth and 1 month, and between 6 and 9 months and complete degradation of mucin starts during the first and



Table 4

*In vitro* methods used to study adhesion of probiotic bacteria to mucus**Sources:**

Mucus or mucus glycoproteins extracted from human faeces (Kirjavainen et al., 1998; Ouwehand et al., 1999), ileostomy glycoproteins (Ouwehand et al., 2001), or pig small intestine (Jonsson et al., 2001).

**Adhesion determination:**

Radioactive labeling of micro-organisms (Cohen & Laux, 1995).

Immobilized mucus in microtiter wells (Jonsson et al., 2001).

Scanning electron microscopy (Sarem-Damerdjji et al., 1995).

Fluorescent-tagged bacteria (Vesterlund et al., 2005).

*In vivo* fluorescence imaging (Francis et al., 2001).

second year of life (Midvedt et al., 1994; Norin et al., 1988). Adhesion may also be reduced by exposure to low pH, suggesting that adherence may be reduced after passage through the stomach (Ouwehand et al., 2001). The health status of an individual also plays an important role. Composition of mucus differs in patients with rectal carcinoma, diverticulitis and irritable bowel syndrome (IBD) (Ouwehand et al., 2003). It is thus safe to assume that the availability of adhesion sites will differ. A decrease in viable cell numbers of lactobacilli and bifidobacteria has been reported in patients with IBD and colon cancer (Salminen et al., 1995). Treatment of these patients with probiotics that have good adhesion properties may alleviate symptoms (Ouwehand et al., 2003). LGG showed better adhesion to tissue from IBD patients than tissue from diverticulitis or rectal carcinoma, whereas *Lactobacillus breve* adhered best to mucus from patients with rectal carcinoma. This study suggested the use of selected strains for specific intestinal diseases. Many other studies (Tables 5-7) show the adhesion of probiotic bacteria to mucus.

No competitive exclusion of *Salmonella typhimurium* or *Escherichia coli* SfaII was observed in the presence of *L. brevis* PEL1, *L. reuteri* ING1, *L. rhamnosus* VTT E-800 and *L. rhamnosus* LC-705 (Ouwehand et al., 2001). Adhesion of S-fimbriated *E. coli* to mucus was relatively high and was slightly reduced by LGG, *L. rhamnosus* LC-705 and *L. rhamnosus* isolated from human faeces. Adhesion of *S. typhimurium* was significantly inhibited by *L. johnsonii* LJ1 and *L. casei* Shirota. However, adhesion of *S. typhimurium* strengthened in the presence of *L. rhamnosus* GG



and *L. rhamnosus* (human isolate), suggesting an interaction between cells (Tuomola et al., 1999).

Table 5

Adhesion of probiotic bacteria to human colonic tissue (obtained from Sarem-Damerdjil et al., 1995)

Probiotic bacteria	Adhesion Bacilli per 1 mm <sup>2</sup> of tissue/ (adhesion evaluation)
Control	0.12 ± 0.06 x 10 <sup>5</sup> (-)
<i>L. acidophilus</i> NCFB 1748	12.96 ± 6.29 x 10 <sup>5</sup> (+)
<i>L. acidophilus</i> Ki	0.15 ± 0.09 x 10 <sup>5</sup> (-)
<i>L. rhamnosus</i> GG	14.32 ± 1.82 x 10 <sup>5</sup> (+)
<i>L. casei</i> 212.3	33.46 ± 8.25 x 10 <sup>5</sup> (++)
<i>L. plantarum</i> 43364	24.75 ± 2.14 x 10 <sup>5</sup> (++)

More than 60 % of probiotic preparations on the market contain strains of enterococci (Franz et al., 1999). Little research has been done on the adhesion of these strains to mucus and their competitive exclusion of pathogens (Franz et al., 1999). The adhesion of *Enterococcus faecium* M74 and *E. faecium* SF68 to mucus was 3 % and 18 %, respectively (Pultz et al., 2006). The adhesion was classified low when compared to 9.2 % adhesion recorded for strain LGG. Approximately 9 % of *Enterococcus faecium* 18C23 cells adhered to small intestine mucus of piglets and effectively inhibited the adhesion of *E. coli* K88ac and K88MB to the mucus. Adhesion of more than 90 % of *E. coli* K88 was inhibited by the addition of 10<sup>9</sup> CFU/ml or higher cell numbers of *E. faecium* 18C23 or pH-neutralized supernatant to mucus. Treatment of mucus with pronase and proteinase reduced the adhesion of *E. coli* K88ac and increased the adhesion *E. faecium* 18C23. The mucus receptors of the two strains may be different and inhibition of *E. coli* K88ac is possibly through steric hindrance (Jin et al., 2000a; 2000b).

Table 6

Adhesion of probiotic bacteria to human ileostomy glycoproteins (obtained from Tuomola et al., 1999, Ouwehand et al., 2001)

Probiotic bacteria	Adhesion (%)
<i>L. rhamnosus</i>	31.6
<i>L. rhamnosus</i> GG	28.7
<i>L. acidophilus</i> LC1	5.6
<i>L. plantarum</i> ATCC 8014	1.7
<i>L. casei</i> (Fyos®)	1.2
<i>L. rhamnosus</i> ATCC 7469	0.74
<i>L. casei</i> Immunitas	0.53
<i>L. casei</i> 01 (Starter culture Chr. Hansen)	0.45
<i>L. casei</i> var. <i>rhamnosus</i> (Lactophilus®)	0.43
<i>L. rhamnosus</i> LC-705	0.41
<i>L. casei</i> (BIO®, Danone)	0.36
<i>L. casei</i> Shirota (Yakult®)	0.35
<i>Lact. lactis</i> subsp. <i>cremoris</i> ARH 74	0.51
<i>L. brevis</i> PEL1	19.3
<i>L. reuteri</i> ING1	36.5
<i>L. rhamnosus</i> E-800	34.0
<i>L. rhamnosus</i> LC-705	0.79
<i>L. rhamnosus</i> GG	35.0

Table 7

Adhesion of probiotic bacteria to mucus of adult humans (obtained from Kirjavainen et al., 1998; Ouwehand et al., 1999)

Probiotic bacteria	Adhesion (%)
<i>L. crispatus</i> Mu5	2.1
<i>L. crispatus</i> M247	10.4
<i>L. rhamnosus</i> GG	46.0
<i>L. johnsonii</i> La1	14.4
<i>L. paracasei</i> F19	9.7
<i>L. salivarius</i> LM2-118	7.7
<i>L. rhamnosus</i> (faecal isolate)	48.0
<i>L. bulgaricus</i>	33.0
<i>L. johnsonii</i> LJ1	30.0
<i>Lc lactis cremoris</i>	23.0
<i>Enterococcus</i>	8.0
<i>L. rhamnosus</i> 744	8.0
<i>L. rhamnosus</i> ATCC 7469	6.0
<i>L. plantarum</i> ATCC 8014	6.0

#### 4.2 Adhesion to epithelial cells

A collection of cell lines was established in the 1970's with the aim of performing studies on cancer mechanisms and related cytostatic therapies (Fogh et al., 1977). Specific intestinal properties of these tumoral cell lines were studied. Addition of synthetic or biological factors induced partial differentiation (Pinto et al., 1983). Caco-2 cells showed a spontaneous differentiation pathway in long-term culture. During differentiation these cells express several morphological and biochemical characteristics of small intestine enterocytes (Pinto et al., 1983). Caco-2 cells have a cylindrical polarized morphology, microvilli on the apical side, tight junction between adjacent cells and express low levels of hydrolase when grown in a monolayer. Expression of these enzymes leads to the selection of prominent sub-populations of cells. Several clonal cell lines have been isolated from the parental line that expresses a homogenous collection of differentiation traits. Results obtained with Caco-2 cell lines in different laboratories are often difficult to compare (Sambuy et al., 2005). Over the past 25 years the Caco-2 cell lines were used extensively in experiments to determine adhesion properties of probiotic bacteria (Sambuy et al., 2005). An alternative *in vitro* adhesion model is the colonic cell line HT-29 which also shows typical characteristics of enterocytic differentiation (Gopal et al., 2001). Other cell lines include fetal I-407 (Gork et al., 1999) and the more recently developed IPEC-J2 isolated from the jejunum of piglets (Schierack et al., 2005). The HT29-MTX cell line from the small intestine of humans was developed to simulate a mucus-secreting environment (Lesuffleur et al., 1990).

Conventional and microscopic methods are used to determine the adhesion of probiotic bacteria to epithelial cell lines (Table 8).

Table 8

*In vitro* methods used to determine the adhesion of probiotic cells to epithelial cell lines

**Conventional and microscopic methods:**

Plate counting after treatment with Triton-X 100 (Bertazzoni-Minelli et al., 2004; Forestier et al., 2001; Shillinger et al., 2005)

Microscopic counting of fixed bacteria after Gram-staining (Tuomola & Salminen, 1998) or Giemsa-staining (Forestier et al., 2001).

Adhesion is calculated as the percentage of bacteria adhered, or adhered bacteria per cell (Shillinger et al., 2005). (These techniques are laborious and bacteria have to remain culturable when released).

Real-time PCR (Candela et al., 2005).

*In vivo* fluorescence imaging (Francis et al., 2001).

**Analysing of adhered bacteria:**

Liquid scintillation counting of radio-labeled bacteria (Gopal et al., 2001).

Fluorescent staining of bacteria and subsequent spectro-fluorimetric detection (Bianchi et al., 2004), especially when using mixed cultures.

Fluorochromes are used to replace the radiolabels because of better safety and cost concerns, but may alter the surface properties of bacteria and affect their viability (Fuller et al., 2000).

Several strains of *L. casei*, *L. paracasei*, *L. acidophilus* and *L. rhamnosus*, isolated from dairy products, were studied for adhesion to cell lines (Baccigalupi et al., 2005; Bertazzoni et al., 2004; Forestier et al., 2001; Gopal et al., 2001; Pennacchia et al., 2006; Shillinger et al., 2005; Tuomola & Salminen, 1998). Adhesion to Caco-2 cells is strain specific and varies from 9.7 % recorded for *L. rhamnosus* GG (Tuomola & Salminen, 1998) to between 3 % and 14 % recorded for other strains of *Lactobacillus* spp. (Tuomola & Salminen, 1998). In the case of mucus-producing HT29 MTX cells, adhesion increased to between 20 % and 40 % (Shillinger et al., 2005). Caco-2 cells have less adhesion sites compared to HT29-MTX cells (Blum et al., 1999). Cell wall structures are expressed from the onset of stationary growth (Blum et al., 1999) and secretion of adhesion promoting factors reach optimal levels towards the end of stationary growth. Structures involved in binding to carbohydrate moieties in mucus are different from structures implicated in adhesion to Caco-2 cells and the process is not influenced by growth phase (Blum et al., 1999). Acetate buffer (pH 5.0) promoted the adhesion of *L. johnsonii* La1, although difference in pH values did not affect the adhesion of *L. rhamnosus* GG (Blum et al., 1999). A study conducted

by Tuomola and Salminen (1998) have shown that commercial probiotic strains with reported health effects such as *L. casei* Shirota did not adhere more effectively than other strains. The authors suggested that cells other than Caco-2 should also be used in adhesion studies. Preliminary studies with Caco-2 cell lines are, however, useful for dose-response studies (Saxelin et al., 1991; 1993; Saxelin, 1997).

Several *in vivo* studies with mice and pigs were conducted to study the adhesion of probiotic strains. Gnotobiotic mice were orally fed with *L. rhamnosus* GG and *L. johnsonii* La1 and the bacteria were established in all segments of the gut (Bernet-Camard et al., 1997). *L. salivarius* and *L. plantarum* strain 299v colonized the gut of gnotobiotic mice (Kabir et al., 1997; Matsumoto et al., 2001). *Lactobacillus* spp. orally administered to gnotobiotic pigs colonized the jejunum and ileum (Bomba et al., 1996). In a few well-designed human trials, colonization of *L. rhamnosus* GG in the intestinal tract was proved by colonic biopsies from patients who consumed a whey drink fermented with the strain (Alander et al., 1997; 1999; Isolauri et al., 1994; Sarem-Damerdjil et al., 1995). *L. johnsonii* La1 and *L. casei* Shirota also survived intestinal transit in adults (Donnet-Hughes et al., 1999). All of these studies showed that probiotics have to be taken on a daily basis to provide a continuous exogenous probiotic effect (Bezkorovainy, 2001).

Intestinal pathogens adhere to the surface of epithelial cells (Alam et al., 1996; Scaletsky et al., 2002; Weinstein et al., 1998). Cell line models have been used to study the mechanisms pathogenic micro-organisms use to cross the intestinal epithelium and infect the host (Chauvière et al., 1992; Forestier et al., 2001; Lee et al., 2003). Lactobacilli have adhesins on their cell surfaces, similar to those on the surface of bacterial pathogens (Neesler et al. 2000). Probiotic strains such as *L. rhamnosus* GG and *L. casei* Shirota may thus compete with intestinal pathogens for adhesion sites in the intestinal tract (Lee et al., 2003). Lactobacilli have the ability to displace gastro-intestinal bacteria. *L. rhamnosus* GG adheres to epithelial cells via hydrophobic interaction and displace a pathogen by steric hindrance. Once bound to the epithelial cells, the bacterial cell is displaced only when it detaches from the enterocytic receptor. *L. rhamnosus* GG inhibited the re-attachment of the bacterium by binding to the enterocytic receptor (Lee et al., 2003). Another example of steric hindrance is the adhesion of heat-killed cells of *L. acidophilus* LB to Caco-2 cells. This inhibited the adhesion of diarrheogenic *E. coli* (Chauvière et al., 1992). Multiple surface adhesins were found on *L. casei* Shirota. Four of these adhesins bound to the

mucosal surface (Lee et al., 2002) and affected competition and exclusion interactions with pathogens. One cell of *L. casei rhamnosus* could out-compete as many as four pathogen cells (Lee et al., 2003). In another study by Forestier et al. (2001) the presence of *L. rhamnosus* (Lcr35) decreased adhesion of three pathogens, enteropathogenic and enterotoxigenic *E. coli* and *Klebsiella pneumoniae*. The access of pathogens to receptor sites was possibly impeded by the addition of Lcr35. A second explanation is the interaction of Lcr35 with the level of mucins produced by Caco-2 cells. Although Caco-2 cells express significant levels of mucins, expression is further elicited in the presence of a probiotic (Mack et al., 1999). Bacteriocins, produced by lactobacilli may also play a role in the competitive exclusion of pathogens. *L. johnsonii* La1 inhibited the growth of *Giardia intestinalis* and its attachment to Caco-2 cells. The factors involved were heat-labile peptides of low molecular mass (Perez et al., 2001). Spent culture supernatant of LB blocked the intracellular life cycle of *S. enterica* serovar Typhimurium SL1344 and inhibited cell damage induced by *Salmonella* and *E. coli* (Coconnier et al., 2000).

Milk with a high fat content reduces the adhesion of probiotic bacteria. Care should therefore be taken during the selection of different fats and fatty acids in the developing of new probiotic food products (Ouweland et al., 2001). A calcium level of 10 mM, which corresponds to the calcium content in milk, increased the adhesion of lactobacilli (Larsen et al., 2007). Adhesion levels of poorly adhesive strains increased when probiotic formulations were supplemented with calcium (Larsen et al., 2007). Polyunsaturated fatty acids possess antimicrobial activities that modulate the action of probiotics in the gut (Kankaanpää et al., 2001). Cellular growth and adhesion of LGG, *L. casei* Shirota and *L. bulgaricus* to mucus were reduced in the presence of high levels (10 - 40  $\mu\text{g PUFA ml}^{-1}$ ) of polyunsaturated fatty acids (PUFA). The adhesion sites on Caco-2 cells were also altered by PUFA. However, low concentrations of  $\gamma$ -linolenic acid and arachidonic acid promoted growth and adhesion of *L. casei* Shirota to mucus. Adhesion of *L. casei* Shirota to Caco-2 cells improved in the presence of  $\alpha$ -linolenic acid (Kankaanpää et al., 2001).

#### 4.3 Mechanisms of adhesion

Mechanisms involved in the binding of probiotic bacteria to epithelial cells and mucus have been studied to understand the balance of intestinal microflora and to provide a basis for rational design of probiotic bacteria (Greene & Klaenhammer, 1994).

Physico-chemical properties of bacteria, which facilitates the first contact between the microorganism and intestinal mucus or epithelial cells, include cell surface hydrophobicity,

passive forces, electrostatic interactions and steric forces, (Schillinger et al., 2005; Wadström et al., 1987). These properties precede the adhesion process mediated by specific mechanisms and are therefore weak and reversible. In contrast to the reports of Wadström et al. (1987), no correlation between hydrophobicity and adhesion to mucus or epithelial cells was observed in subsequent studies (Ouwehand et al., 1999; Savage, 1992; Shillinger et al., 2005). These discrepancies can be explained by the use of different methods (Ouwehand et al., 1999; Savage, 1992; Shillinger et al. 2005). Although hydrophobicity might assist adhesion, it is not a prerequisite for strong binding to mucus and epithelial cells (Shillinger et al., 2005).

Greene and Klaenhammer (1994) pre-treated *Lactobacillus* spp. with proteases of the human gastro-intestinal tract and periodate to examine variations in proteins and carbohydrate adhesion sites on the cell walls. The adhesion process involves specific mechanisms that include cell-surface proteins (Adlerberth et al., 1996; Bernet et al., 1994; Roos & Jonsson, 2002; Satoh et al., 1999), carbohydrates (Granato et al., 1999; Vidal et al., 2002), hemagglutinins (Andreu et al., 1995), aggregation promoting factors (Ventura et al., 2002), S-layer proteins (Frece et al., 2005) and lipoteichoic acids (Greene & Klaenhammer, 1994). Various proteins involved in adhesion of *Lactobacillus* spp. have been isolated and characterized (Table 9).

Many bacteria have a crystalline layer on their cell surface, which changes with the environment (Boot & Powels, 1996). This layer consists of single protein or glycoprotein species with relative molecular weights of 40 000 to 200 000, representing 10-15 % of the total protein of the bacterial cell (Sára & Sleytr, 2000). Bacteria have efficient expression of genes, synthesis and secretion of S-layer proteins (Boot & Pouwels, 1996). The S-layer structure contains pores of identical size and morphology that comprise up to 70 % of the lattice surface area (Sleytr & Beveridge, 1999). S-layer proteins are involved in cell protection, adhesion, trapping molecules and ions, and virulence (Åvall-Jääskeläinen & Palva, 2005; Sleytr & Jacobsen, 1983). S-layer proteins isolated from *L. acidophilus*, *L. amylovorus*, *L. brevis*, *L. buchneri*, *L. casei*, *L. crispatus*, *L. fermentum*, *L. gallinarum*, *L. helveticus*, *L. kefir*, *L. parakefir* vary from 25 to 71kDa in size (Åvall-Jääskeläinen et al., 2002; Barker & Boot et al., 1993; 1995; 1996; Callegari et al., 1998; Jakava-Viljanen et al., 2002; Kos et al., 2003; Lortal et al., 1992; Masuda & Ka-wata, 1981; 1983; Sillanpää et al., 2000; Thorne, 1970; Vidgrén et al., 1992).

Table 9

Adhesion factors isolated from *Lactobacillus* spp.

Bacterial strains	Adhesion factors	Characterization	Molecular mass (kDa)	References
<i>L. plantarum</i> 299, <i>L. plantarum</i> 299v	Mannose-specific adhesin	Cell-surface protein	-	Adlerberth et al. (1996)
<i>L. johnsonii</i> NCC533 (La1)	LTA	Cell Surface-Associated lipoteichoic acid	46	Granato et al. (1999)
<i>L. gasseri</i> SBT267	SLP	Cell-surface lectin	50	Matsumura et al. (1999)
<i>L. gasseri</i> LA187			30	
<i>L. gasseri</i> LA2			25	
<i>L. reuteri</i> 104R	MapA	Cell-surface protein, mucus adhesion promoting protein	30	
<i>L. reuteri</i> 1063	Mub	Cell-surface protein	26	Satoh et al. (1999)
<i>L. gasseri</i> VPI 11759	Apf1	Aggregation promoting factor	358	Roos & Jonsson, (2002) Ventura et al. (2002)
<i>L. gasseri</i> ATCC 19992	Apf2		29.8	
<i>L. johnsonii</i> ATCC 332,	Apf1		29.8	
ATCC 533(La1)	Apf2		26.3	
ATCC 33200	Apf1		26.3	
ATCC 20553	Apf2		26.3	
DSM 11506	Apf1		27.45	
<i>L. johnsonii</i> NCC533 (La1)	EF-Tu	Cell Surface-Associated Elongation factor Tu	31.0	



Many of the genes encoding these proteins have been sequenced (Table 10). S-layer proteins in lactobacilli function in adhesion to epithelial cells (Frece et al., 2005; Schneitz et al., 1993) and mammalian extracellular matrix (stable macromolecular structure underlying epithelial and endothelial cells) (Hynonen et al., 2002; Sillanpää et al., 2000; Toba et al., 1995). S-layer proteins of *L. crispatus* ZJ001 are involved in adhesion to epithelial cells and competitive exclusion of pathogens such as *Escherichia coli* O157:H7 and *Salmonella typhimurium* (Chen et al., 2007). However, removal of the S-layer proteins by treatment with LiCl revealed that they are not involved in the adhesion of lactobacilli to Caco-2 cells (Greene & Klaenhammer, 1994).

Table 10

Sequenced S-layer protein genes, isolated from *Lactobacillus* species (obtained from Åvall-Jääskeläinen & Palva, 1995)

Bacterial species	Strain	GenBank accession number
<i>L. acidophilus</i>	ATCC 4356	AF250229
<i>L. crispatus</i>	JCM 5810	AB110090
		AB110091
		AJ007839
	LMG 12003	AF253043
		AF253044
<i>L. helveticus</i>	GCL1001	AB061775
	JCM1003	AB061776
	ATCC 12046	AJ388558
	ATCC 15009	AJ388559
	CNRZ 303	AJ388560
	CNRZ 35	AJ388561
	IMPC i60	AJ388562
	IMPC M696	AJ388563
	IMPC HLMI	AJ388564

### 5. *In vitro* and *in vivo* fluorescent imaging

Fluorescent staining is a safe and cost-effective technique to visualize bacteria *in vitro* (Fuller et al., 2000). Lectin binds specifically to *N*-acetylglucosamine in the peptidoglycan layer of Gram-positive bacteria. Cell-permeating nucleic acid stains, e.g. intercalating dyes such as ethidium bromide and propidium iodide and minor-groove binders such as DAPI and Hoechst dyes, stain Gram-negative bacteria (Mason et al., 1998).

Vesterlund and co-workers (2005) described a new model which involves the use of resected human intestinal tissue, fluorescent-tagged bacteria and confocal laser scanning microscopy (CLSM), as well as human intestinal mucus and bioluminescent-tagged bacteria. Fluorescent protein encoding genes are transformed into bacteria to ensure minimal background fluorescence. Fluorescent-tagged bacteria can be detected in an environment where other bacteria are present.

Animal models are used in preclinical tests to evaluate therapeutic agents. These tests are time consuming and do not always give a true prediction of the outcome of human clinical trials. Real-time *in vivo* imaging requires fewer test animals and less time than other animal testing models. Only one animal is needed to collect temporal and spatial data over different time points (Sadikot & Blackwell, 2005).

A bioluminescent or fluorescent reporter gene is expressed in a living organism and real-time *in vivo* imaging utilizes the light emitted. The strength of the signal is analyzed and allows extensive three-dimensional modeling in the live animal. Cellular or genetic activity is monitored to track gene expression, the spread of a disease or the effect(s) of a new potential drug *in vivo* (Sadikot & Blackwell, 2005).

A few studies have been done using *in vivo* fluorescence imaging. A novel Gram-positive *lux* transposon cassette, Tn4001 *lux*ABCDE Km<sup>r</sup> allows random integration of *lux* genes onto the genome of bacteria. Clinical *Streptococcus pneumoniae* isolates were transformed with plasmid Tn4001 *lux*ABCDE Km<sup>r</sup>, in a pneumococcal lung infection model for drug development. The outcome of antibiotic treatment could be monitored in real time in living animals. Strong bioluminescent signals were seen in the lungs of the infected animals and corresponded with recovered bacteria (Francis et al., 2001). The effectiveness of amoxicillin treatment in infection of mice was determined by transforming strains of *Staphylococcus aureus* with plasmid DNA containing a *Photobacterium luminescens lux* operon (*lux*ABCDE). The light signal decreased after 8 h and no signal was detected after 24 h, which indicated that the antibiotic was effective (Francis et al., 2000). Three different antibiotics were evaluated for antimicrobial activity in a mouse model. The metabolic activity of rifampin was dose-dependent and the disappearance of light emission correlated with decrease in colony counts (Kadugamuwa et al., 2003). These studies are examples of successful *in vivo* fluorescence imaging. *In vivo* monitoring has numerous other applications. Probiotic bacteria can be monitored in the gastro-intestinal tract to

determine their survival, adhesion and competitive exclusion of pathogens causing infection and application of these techniques will ensure high quality probiotic research in the future.

## **6. Characterization of antimicrobial agents produced by LAB**

Fermentation of carbohydrates by LAB leads to the production of antimicrobial compounds including organic acids, diacetyl, hydrogen peroxide or other low molecular weight compounds such as reuterin (Piard & Desmazeaud, 1992a; 1992b; Vandenberg, 1993). Antimicrobial activity of LAB could also be due to the production of bacteriocins, a family of antimicrobial peptides that have gained increasing interest. These substances exert in some cases a narrow, or in other cases, a relatively broad spectrum of antimicrobial activity (Rodríguez et al., 2003).

### *6.1 Organic acids*

Lactic acid is produced during homofermentation of hexoses and a combination of lactic acid, acetic acid/ethanol and CO<sub>2</sub> by heterofermentation (Ouweland & Vesterlund, 2004). Acetic and propionic acid have a wide spectrum of inhibitory activity, which includes yeasts, molds, and bacteria (Ouweland & Vesterlund, 2004). In a mixture, the lactic acid reduces the pH, permeabilizes membranes and hereby enhances the activity of the propionic and acetic acids which become undissociated and act as antimicrobial agents (Alakomi et al., 2000). The acid diffuses across the cell membrane, dissociates in the neutral cytoplasm, releasing protons and anions which lead to growth inhibition (Cherrington et al., 1991; Padan et al., 1981; Slonczewski et al., 1981).

### *6.2 Hydrogen peroxide*

LAB generate hydrogen peroxide that may scavenge oxygen and create an anaerobic environment that is unfavorable for certain microorganisms (Ouweland & Vesterlund, 2004). Gram-negative bacteria are rapidly killed, whereas the growth of Gram-positive bacteria including LAB is inhibited (Blom & Mørtvedt, 1991; Condon, 1987; Lindgren & Dobrogosz, 1990). Hydrogen peroxide has a strong oxidizing effect on the bacterial cell, sulfhydryl groups of cell proteins and membrane lipids (Morris, 1976; Schlegel, 1985; Lindgren & Dobrogosz, 1990). Lactobacilli, which colonize the urogenital tract, produce hydrogen peroxide and decrease the acquisition of human immune deficiency virus (HIV) infection, gonorrhea and urinary tract infections (Vallor et al., 2001). The main antimicrobial effect is the blocking of glycolysis. Glucose transport, hexokinase activity, and glyceraldehyde-3-phosphate dehydrogenase activity

are inhibited due to the oxidation of sulfhydryl groups in metabolic enzymes (Carlsson et al., 1983).

### 6.3 Carbon dioxide

Production of carbon dioxide (CO<sub>2</sub>) during lactic acid fermentation creates a micro-aerophilic environment. The accumulation of enzymatic decarboxylations in the lipid bilayer is inhibited which causes dysfunction in membrane permeability (Lindgren & Dobrogosz, 1990; King et al., 1975). Gram-negative bacteria are more sensitive to CO<sub>2</sub> than Gram-positive bacteria (Devlieghere & Debevere, 2000).

### 6.4 Diacetyl

Diacetyl (2,3-butanedione) produced by *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* spp. is not only the aroma and flavor component in butter but has antimicrobial activity (Jay, 1982; Lemoigne, 1927). Diacetyl reacts with the arginine-binding protein of Gram-negative bacteria and interferes with arginine utilization (Jay, 1982). Diacetyl is more active against Gram-negative bacteria, yeasts and molds than against Gram-positive bacteria (Jay, 1982).

### 6.5 Reuterin

*Lactobacillus reuteri* produces a low molecular-weight antimicrobial substance, reuterin, when grown anaerobically in the presence of glucose and glycerol or glyceraldehydes (Axelsson, 1989). When in contact with target cells, *L. reuteri* is stimulated to produce reuterin which is active against bacteria, fungi, protozoa and viruses (Axelsson, 1989; Chung et al., 1989; Dobrogosz et al., 1989). Reuterin acts against sulfhydryl enzymes and interferes with DNA-synthesis by inhibiting the binding of substrates to the subunit of ribonucleotide reductase (Dobrogosz et al., 1989).

### 6.6 Reutericyclin

*L. reuteri* produces another antimicrobial substance, reutericyclin with a low molecular weight (349 Da), a negative charge and high hydrophobicity (Höltzel et al., 2000). Only Gram-positive bacteria are sensitive to reutericyclin (Gänzle et al., 2000). It works as a proton iophore, forming pores in the membranes of target cells. Due to its hydrophobicity, it intercalates into the cytoplasmic membrane and selectively dissipates the transmembrane  $\Delta$ pH (Gänzle et al., 2003).

### 6.7 2-Pyrrolidone-5-carboxylic acid

Pyroglutamic acid (PCA) is present in fruits, vegetables and grasses and is produced by *Lactobacillus casei* subsp. *casei*, *L. casei* subsp. *pseudopiantarum* and *Streptococcus bovis* (Chen & Russell, 1989; Huttunen et al., 1995). Pyroglutamic acid has a stronger antimicrobial activity than lactic acid and its mechanism of action is similar to that of organic acids and inhibits *Bacillus subtilis*, *Enterobacter cloacae*, *Pseudomonas putida* and *Pseudomonas fluorescens* (Yang et al., 1997).

### 6.8 Bacteriocins

Some bacteria produce antimicrobial peptides (namely bacteriocins) inhibitory to strains closely related to the producer strain (Klaenhammer, 1988; Tagg et al., 1976). Since the discovery of bacteriocins the interest in such compounds as possible preservative agents for food, and potential supplements or as replacements for therapeutic antibiotics has increased (Ouwehand & Vesterlund, 2004). Consumer awareness of bacteriocins has increased as they are naturally produced and therefore more easily accepted than chemical preservatives (Ouwehand & Vesterlund, 2004). Gram-positive bacteria, especially LAB, produce these small (2-6 kDa) peptides ribosomally as a defense mechanism against other organisms (Hansen et al., 1989). Gram-positive bacteria produce novel bacteriocins in competitive environments resulting in peptides bearing unique structural features and varied modes of action. Bacteriocins are divided into three major classes (Table 11). A fourth class (complex bacteriocins with carbohydrates or lipids) was suggested, but is not generally accepted (Ouwehand & Vesterlund, 2004). Enterocins are produced by *Enterococcus faecium*, *E. faecalis* and *E. mundtii* and can also be grouped into one of these classes. Enterocins such as enterocin A, enterocin CRL35, enterocin 31 and enterocin B with unusual structures or genetic characteristics can not be grouped into this classification scheme (Franz & Holzapfel, 2004).

Table 11

Classes of bacteriocins produced by LAB (Ouwehand &amp; Vesterlund, 2004)

Class	Subclass	Description
I (lantibiotics)	A (1)	Elongated, cationic, membrane active, slight positive or negative net charge
	A(2)	Elongated, cationic, membrane active, highly positive net charge
	B	Globular, inhibit enzyme activity.
II		Small 9<10kDa), moderate (100°C) to high (121°C) heat stable, non-lanthionine-containing membrane-active peptides.
	IIa	Listeria active peptides with –Y-G-N-G-V-X-C-near the amino terminus.
	IIb	Two peptide bacteriocins
	IIc	Other peptide bacteriocins
III		Large (>10 kDa) heat-labile proteins
IV		Complex bacteriocins: protein with lipid and/or carbohydrate.

### 6.8.1 Class I

Class I bacteriocins (lantibiotics) are small (< 5 kDa) peptides with dehydrated amino acids and unusual amino acids that are synthesized during posttranslational modifications (Jack & Sahl, 1995). The two groups of lantibiotics (types A and B) differ in structure and function. The elongated cation peptides of type A disrupt the membrane integrity of target organisms. Type A is further classified into two groups on the basis of size, charge, and sequence of the leader peptide (De Vos et al., 1995). The globular type B disrupts enzyme function by inhibiting the biosynthesis of the cell wall (McAuliffe et al., 2001). A well-known and widely used commercial lantibiotic is nisin. Nisin is produced by *Lactococcus lactis*, has a broad spectrum of activity against Gram-positive bacteria and is bactericidal to most LAB, *Staphylococcus aureus*, *Listeria monocytogenes*, vegetative cells of *Bacillus* spp. and *Clostridium* spp. (Klaenhammer, 1993). Nisin forms pores in the cytoplasmic membrane of bacteria by using lipid II as a docking molecule (Breukink et al., 1999). Small compounds, such as amino acids and ATP, diffuse

through these pores, resulting in disruption of the proton-motive force (PMF). The biosynthesis of macromolecules, e.g. DNA, RNA and proteins is inhibited (Hechard & Sahl, 2002). In addition, nisin can lyse cells by releasing two strongly cationic cell wall-hydrolyzing enzymes (*N*-acetylmuramoyl-L-alanine amide and *N*-acetylglucosaminidase) through a cation exchange-like process (Hechard & Sahl, 2002). Cytolysin is the only known class I enterocin which has bacteriocin and haemolytic activity against eukaryotic cells and Gram-positive bacteria (Booth et al., 1996; Gilmore et al., 1994).

### 6.8.2 Class II

These small (<10 kDa) bacteriocins consist of heat-stable, non-lanthionine-containing, membrane-active peptides with a narrow inhibition spectrum (Ouwehand & Vesterlund, 2004). The three subclasses include Class IIa, the largest group with antilisterial activity. Class IIb includes the two peptide-bacteriocins and Class IIc the other non-lantibiotic bacteriocins (Eijsink et al., 2002; Hechard & Sahl, 2002). These bacteriocins dissipate the PMF of the target cell by forming pores at the surface of the sensitive cell (Hechard & Sahl, 2002). Some class IIc bacteriocins can prevent septum formation (Matinez et al., 2000).

Class IIa bacteriocins are used to improve food safety and reduce the prevalence of foodborne illnesses because they are highly active against *Listeria* spp. Pediocin PA-1, produced by *Pediococcus acidilactici*, is the most extensively studied from this class and is encoded by an operon containing a structural gene (*pedA*), immunity gene (*pedB*) and secretion genes (*pedC* and *pedD*) (Ouwehand & Vesterlund, 2004). Enterocin A (EntA) produced by *E. faecium* strains, is a class IIa enterocin as it is also active against *L. monocytogenes*. Mundticin KS is another class IIa enterocin produced by *Enterococcus mundtii* NFRI 7393, active against *L. monocytogenes*, *Lactobacillus* spp. and *Enterococcus* spp. (Kawamoto et al., 2002). Class IIb enterocins include enterocin 1071 and enterocin L50 produced by strains of *E. faecalis* and *E. faecium*, respectively, and have a broad range of antimicrobial activity (Balla et al., 2000; Franz et al., 2002; Nes et al., 1996). Enterocin P and enterocin 1071 produced by *E. faecium*, and bacteriocin 31 produced by *E. faecalis*, are classified as class IIc enterocins (Balla et al., 2000; Casaus et al., 1997; Tomita et al., 1996).

### 6.8.3 Class III

Class III bacteriocins are large (>30kDa) heat-labile proteins and may include bacteriolytic extracellular enzymes (hemolysins and muramidases) which mimic the physiological activities of bacteriocins (Jack et al., 1994). These bacteriocins were only isolated from lactobacilli and include acidophilucin A, caseicin, helveticin J, helveticin V-1829, lactacin A and lactacin B (Klaenhammer, 1993). The only enterocin grouped in class III is enterolysin A produced by *E. faecalis* LMG 2333. Enterolysin A has a molecular weight of 34 501 Da, is heat-labile and inhibits growth of *Enterococcus*, *Pediococcus*, *Lactococcus* and *Lactobacillus* spp. (Nilsen et al., 2003).

### 6.8.4 Class IV

This class contains complex bacteriocins with lipid or carbohydrate moieties which are necessary for activity. Limited data is available for this class and it is not discussed further (Klaenhammer, 1993).

Atypical enterocins cannot be grouped into any of the four classes. Examples are enterocins B and Q, produced by *E. faecium*, and enterocin EJ97 and AS-48, produced by *E. faecalis* spp. (Aymerich et al., 1996; Casaus et al., 1997; Gálvez et al., 1986; Gálvez et al., 1998; Papadelli et al., 2003; Sánchez-Hidalgo et al., 2003).

Some of the different methods used to determine antimicrobial activity of bacteria are summarized in Table 12. The production of antimicrobial agents by lactobacilli to inhibit adhesion and infection of pathogens is illustrated in Figure 5.



Table 12

Methods for studying antimicrobial activity

### Agar well diffusion assay (Cintas et al., 1995)

Cell-free supernatants of bacteriocin producers with neutralized pH are used.

The indicator strain is plated onto agar plates and the supernatant is spotted onto the agar or into wells in the agar. Agar plates are screened for clear zones which indicate positive results.

### Cell lysis (Ouwehand & Vesterlund, 2004)

Incubate target strain with cell-free culture supernatant. Determine cell density or viable cell numbers.

### Fluorescence (Budde & Rasch, 2001)

Bacteriocins are stained with carboxyfluorescein diacetate and exposed to the target organism. Fluorescence is measured with flow cytometry. A decrease in fluorescence indicates the effect of the bacteriocin.

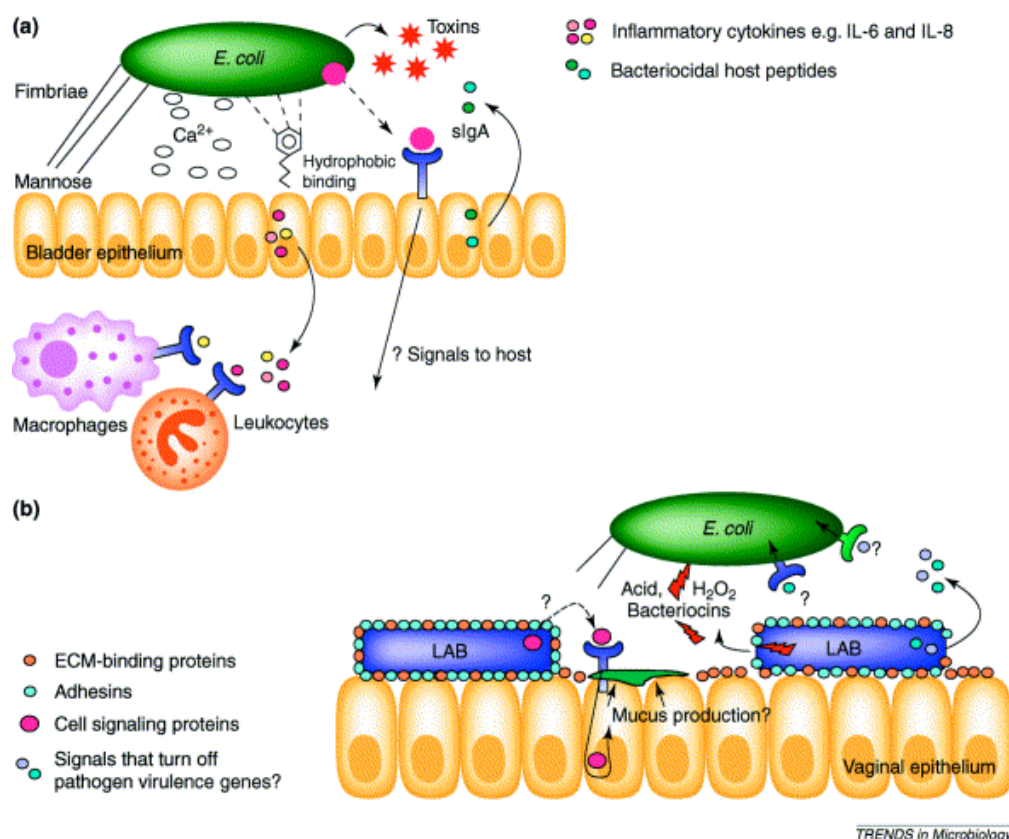


Fig. 5. (a) The process whereby a pathogen, in this case uropathogenic *Escherichia coli*, adheres to the bladder surface using various mechanisms, and causes inflammation and infection. (b)

Lactobacilli colonization of the vagina interferes with pathogen adhesion and infection. The ability of *E. coli* to dominate the flora and ascend into the bladder is reduced by lactobacilli. In the case of a vaginal pathogen, such as *Gardnerella vaginalis*, lactobacilli interfere with the infectious process at the epithelial interface. The signaling molecules produced by commensal or exogenously applied probiotic organisms activate the host (e.g. by stimulating mucus production) and/or inhibit the response of the pathogen (e.g. activation of their virulence genes). Abbreviations: ECM, extracellular matrix; IL, interleukin; slgA, secretory IgA (Reid et al., 2001).

## 7. Safety of probiotic bacteria

### 7.1 *Lactobacillus* spp.

Lactic acid bacteria have a long history in the dairy industry and are generally regarded as safe (GRAS). For more than 70 years, species such as *L. acidophilus* were used as probiotics (Salminen et al., 1998b). Caution should be taken in the cases of immunosuppressed patients as individual cases of infection have been reported (Vesterlund et al., 2007). Other cases of infection caused by non-probiotic lactobacilli were also reported (Table 13). Cannon et al. (2005) reported 241 cases and Husni et al. (1997) 45 cases of infection. Bacteremia was the most common infection associated with *Lactobacillus*, especially *L. casei* and *L. rhamnosus*, followed by endocarditis and localized infections (Cannon et al., 2005). Safety of new potential probiotic species cannot be assumed and assessment of individual strains is important (Saxelin et al., 2005).

Safety assessment of a probiotic strain includes studies of the intrinsic properties and pharmacokinetics of the strain (survival and growth in the intestine, dose-response relationships, and fecal and mucosal recovery) and interactions between the strain and host (Salminen et al., 1998b). Enzymatic properties, e.g. excessive deconjugation of bile salts, degradation of mucus, platelet aggregating and enzymes favoring cardiac valve colonization are studied *in vitro* (Donohue et al., 1998; Korpela et al., 1997; Pelletier et al., 1996). The pharmacokinetics of probiotics is measured *in vivo* using a fecal collection of intestinal intubation and colonic biopsy techniques (Alander et al., 1997; Johansson et al., 1993; Saxelin, 1996). It is much more difficult to predict illness related to microbiological agents in

Table 13

Classification of probiotic organisms (Donohue & Salminen, 1996; Gasser, 1994; Vesterlund et al., 2007)

Organism	Infection potential
<i>Lactobacillus</i>	Mainly non-pathogens, some opportunistic infections (usually in immunocompromised patients)
<i>Lactococcus</i>	Mainly non-pathogens
<i>Leuconostoc</i>	Mainly non-pathogens, some isolated cases of infection
<i>Streptococcus</i>	Oral streptococci mainly non-pathogens (including <i>Streptococcus salivarius</i> ), some may cause opportunistic infections
<i>Enterococcus</i>	Some species are opportunistic pathogens with haemolytic activity and antibiotic resistance
<i>Bifidobacterium</i>	Mainly non-pathogens, some isolated cases of human infection
<i>Saccharomyces</i>	Mainly non-pathogens, some isolated cases of human infection

food than illness caused by chemical agents (Tang et al., 1993). Differences in microbial and host factors make it difficult to determine minimum dosages (Salminen et al., 1998a).

The capacity of probiotic bacteria to invade epithelial cells is studied by culturing intestinal cells (Tang et al., 1993). Translocation of probiotic bacteria, mucus degradation and immunological side-effects are determined in animal models (Salminen et al., 1998b). Short-term human trials are also used to assess the safety of well-known probiotics. This has been done for *L. rhamnosus* GG, *L. johnsonii* LC1, *L. acidophilus* NCO 1748 and *L. casei* Shirota (Lidbeck et al., 1987, 1988; Orrhage et al., 1994; Saxelin, 1997).

Four types of side-effects exist, namely systemic infections, deleterious metabolic activity, excessive immune stimulation and risk of gene transfer (Salminen et al., 1998b). Local or systemic infections, including septicemia and endocarditis, are rarely caused by lactobacilli and bifidobacteria and are mostly associated with the host's own microflora (Salminen et al., 1998b). The most dominant bacteria colonizing the small intestine may induce diarrhea and cause intestinal lesions by deconjugation and dehydroxylation of bile salts (Donohue et al., 1998). Possible side-effects of probiotics with high BSH-activity are now being investigated (Salminen et al., 1998b). Strains that metabolize glycoproteins and synthesize and lyse fibrin clots which

lead to enhanced infection risk of endocarditis, should not be considered for use as probiotics (Oakley et al., 1995). No immunological side-effects of any probiotic were reported in humans (Salminen et al., 1998b). Some strains of *L. casei* administered parenterally induced side-effects such as fever, arthritis, and hepatobiliary lesions (Schwab, 1993). Cell wall components, e.g. peptidoglycans, elicit cytokines which mediate these side-effects (Miettinen et al., 1996; Perdigon et al., 1991).

*Lactobacillus* and *Bifidobacteria* prevent gastro-intestinal side effects after antibiotic administration by restoring the intestinal microflora (Courvalin, 2006). The spread of antibiotic resistance genes to pathogenic bacteria has been discovered recently. This has led to the concern about the use of multidrug resistant probiotics and their impact on the antibiotic resistance patterns of bacteria causing diseases (Courvalin, 2006).

Two types of bacterial resistance to antibiotics exist, namely intrinsic and acquired resistance (Courvalin & Trieu-Cuot, 2001). Intrinsic, or natural, resistance, is present in all strains of a given genus or species. It defines the spectrum of activity of an antibiotic and is chromosomal and not transferable to other bacteria. Acquired resistance is present only in a certain number of bacteria belonging to a specific genus or species and is induced by mutations in indigenous (house keeping) genes. Acquisition of an exogenous resistance determinant from another bacterium is conveyed by horizontal (lateral) gene transfer (Courvalin, 2006). The genetic changes alter the defensive functions of the bacteria by changing the target of the drug, i.e. by changing the membrane permeability, enzymatic inactivation of antibiotics (e.g.  $\beta$ -lactamases, aminoglycoside acetyl-, nucleotidyl- and phosphoryl-transferases), active transport of antibiotics (e.g. by membrane inserted ATP-dependent efflux systems), target modification (e.g. methylation of 23S rRNA, mutation of amino acid sequence of topoisomerase) (Davies, 1997), or routing metabolic pathways around the disrupted point (Poole, 2002).

Lactobacilli, pediococci and *Leuconostoc* spp. have a high natural resistance to vancomycin, which is different for other Gram-positive bacteria (Hamilton-Miller & Shah, 1998; Simpson et al., 1988). Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin (Danielsen & Wind, 2003). In the case of *Lactobacillus* spp., susceptibility to antimicrobial agents is species-

dependent (Danielsen & Wind, 2003). The antibiotic resistance profile of 55 probiotics used in Europe revealed resistance against kanamycin (79% of the isolates), vancomycin (65 %), tetracycline (26 %), penicillin G (23 %), erythromycin (16 %) and chloramphenicol (11 %). Overall 68.4 % of the isolates showed resistance to multiple antibiotics (Temmerman et al., 2002). Growth of probiotic bacteria is inhibited by the majority of antibiotics. In the case of multiresistance, the probiotic can be co-administrated with antibiotics. The possible negative effects are firstly the transfer of genes encoding resistance, directly or indirectly via the commensal flora from the probiotic to human bacterial pathogens. Secondly, only a limited number of antibiotics can be used to treat a patient when probiotics acquired resistance genes from human commensals or from multiresistant probiotics that caused infection in immunocompromised patients (Courvalin, 2006).

Lactic acid bacteria, like all other bacteria, acquire genes to ensure their survival in antibiotic containing environments. Food microbiologists should take care to avoid the distribution of bacteria with mobilizable antibiotic resistances (WHO, 1997). Special purpose probiotics have been developed to use in combination with antibiotics through the introduction of multiple resistance to the bacteria. However, probiotics should not carry genes other than those required to prevent the undesirable transfer of resistance or conferment of resistance to endogenous bacteria (Mathur & Singh, 2005).

## 7.2 *Enterococci*

*Enterococcus* spp. can also be used as probiotics and a few strains have been studied in clinical trials. *E. faecium* SF68, produced in Switzerland, is effective in the prevention and treatment of diarrhea and can be used as an alternative to antibiotic treatment (Lewenstein et al., 1979; Bellomo et al., 1980). A fermented milk product containing this strain is also sold in Denmark for its hypercholesterolemia effect (Agerbaek et al., 1995). A fermented soymilk drink has been developed by using *E. faecium* CRL 183. Cholesterol decreased by 43% *in vitro* (Rossi et al., 1999). The probiotic strain, *E. faecium* Fargo 688® alleviated the symptoms of irritable bowel syndrome in a clinical trial and was successfully used in the production of cheddar cheese (Allen et al., 1996; Gardiner et al., 1999). The scientific committee on animal nutrition classified *E. faecium* DSM 7134, NCIMB 10415, CECT 4515, NCIMB 30098, NCIMB 1181, DSM 5464, DSM 3520, NCIMB 10415, DSM 4788, DSM 4789 and *E. mundtii* CNCM MA 27/4E safe to use as probiotics in animals (European commission, 2004).

Despite a few preliminary reports on the safety of *Enterococcus* spp. as probiotics, controversy still exists in the use of these organisms as probiotics (Lewenstein et al., 1979). One of the main reasons for this is the association of enterococci with human diseases and multiple antibiotic resistances. Bacteremia, endocarditis, urinary tract and other infections, especially in immunocompromised patients, or those with severe underlying disease, are among the human diseases caused by enterococci (Morrison et al., 1997; Murray, 1990; Linden & Miller, 1999). *E. faecalis* is the most dominant enterococcus in human infections (more than 80 %). *E. faecium* is associated with the majority of remaining infections (Jett et al., 1994) and is the causative agent in enterococcal bacteremia probably because of vancomycin- resistance (Mundy et al., 2000).

Studies have shown that antimicrobial resistance genes and genes encoding virulence factors can be transferred to other bacteria in the gastro-intestinal tract (Franz & Holzapfel, 2004). Enterococci are resistant to a wide variety of antibiotics that contributes to pathogenesis (Landman & Quale, 1997; Leclercq, 1997; Murray, 1990). Intrinsic resistance occurs to the cephalosporins,  $\beta$ -lactams, sulphonamides, and low levels of clindamycin and aminoglycosides (Leclercq, 1997; Moellering, 1990; Moellering, 1992; Morrison et al., 1997; Murray, 1990). Acquired resistance, based on acquisition of plasmids and transposons, include chloramphenicol, erythromycin, high levels of clindamycin, aminoglycosides, tetracycline,  $\beta$ -lactams (by  $\beta$ -lactamase or penicillinase), fluoroquinolones and glycopeptides (Landman & Quale, 1997; Leclercq, 1997; Moellering, 1991; Morrison et al., 1997; Murray, 1990).

Vancomycin is of special concern as it is used for treatment of multiple drug resistant infections. In the mid-1990s vancomycin-resistant enterococci (VRE) were isolated from farm animals. This led to the transmission of VRE through food in hospitals and infections that cannot be treated with conventional antibiotic therapy (Franz et al., 2003). Vancomycin resistant enterococci possess *vanA*, *vanB*, *vanC1*, *vanC2*, *vanC3*, *vanD* and *vanE* type resistance genes (Arthur & Courvalin, 1993; Leclercq, 1997).

Glycopeptide resistant enterococci are phenotypically and genotypically heterogeneous. VanA and VanB are the most frequently observed in two predominant enterococcal species (Arthur & Courvalin, 1993). The VanA-type confers high level inducible resistance to vancomycin and teicoplanin and VanB-type displays variable levels of inducible resistance. A third type, VanC, occurs in enterococci with low-level resistance to vancomycin. The VanC-type is also an intrinsic property of the motile species *E. gallinarum*, *E. casseliflavus* and *E. flavescens* (Dutka-

Malen et al., 1995; Leclercq, 1997; Vincent et al., 1991). Only a few alternatives remain for successful treatment of VRE (Fraise, 1996) as they are highly resistant to all standard anti-enterococcal drugs, including penicillin–aminoglycoside combinations (Landman & Quale, 1997). Therefore, VRE presently constitute a serious risk among bacterial nosocomial pathogens and their presence in hospitals is met with great concern (Franz et al., 1999).

The virulence of enterococci involves more than antibiotic resistance and includes a number of events, e.g. colonization of and adhesion to host tissues, invasion of the tissue and resistance to both nonspecific and specific defense mechanisms mobilized by the host (Franz & Holzapfel, 2004). Pathological changes are produced by the pathogen by toxin production or indirectly by inflammation (Johnson, 1994). Virulence traits have been determined in clinical isolates that may be associated with one or more stages of infection (Franz et al., 2003; Jett et al., 1994).

### 7.3 Virulence factors

#### 7.3.1 Aggregation substance (AS)

The production of aggregation substance (encoded by *E. faecalis* pheromone-responsive plasmids), is induced by small (7-8 amino acids) hydrophobic peptides, named sex pheromones (Franz et al., 2003). Efficient enterococcal donor-recipient contact is mediated by AS to facilitate plasmid transfer (Clewell, 1993). This might contribute to pathogenesis of infection by enterococci through different mechanisms (Foulquié Moreno et al., 2006). Aggregation substance is also involved in binding to eukaryotic cells and extracellular matrix (ECM) proteins e.g. fibronectin, thrombospondin, vitronectin and collagen type I (Rozdzinski et al., 2001). Penetration of the intestinal or genitourinary epithelium to the lymphatic and/or vascular system by enterococci causes abdominal infection and bacteremia (Graninger and Ragette, 1992; Wells et al., 1988). The translocation of enterococci through intact epithelial cell layers was reported in a few studies. Aggregation substance is the only virulence factor which plays a role in translocation of enterococci. The bacteria encounter the basal membrane and ECM. Adhesion to exposed extracellular proteins is promoted in cases of intestinal lesions which lead to the promotion of bacterial translocation (Franz et al., 2003). Adhesion to ECM proteins might also play a role in wound infections and bacterial endocarditis (Benn et al., 1997; Rozdzinski et al., 2001). Aggregation substance plays a role in increased adhesion to human neutrophils (PMNs) and macrophages, leading to increased survival in immune cells. The phagosomes in activated human neutrophils contained AS-bearing enterococci which were much larger than phagosomes



containing opsonized *E. faecalis*. Modification of phagosomal maturation is involved in AS-induced resistance to killing (Rakita et al., 1999).

### 7.3.2 Sex pheromones

Sex pheromones are cleavage products of 21- to 22-amino-acid signal peptides which are associated with lipo-proteins and considered as virulence determinants (Clewett et al., 2000). These pheromones and their surface exclusion proteins cause pathological changes including acute inflammation. They induce the production of superoxide, secretion of lysozyme enzymes and are chemotactic for human and rat PMNs *in vitro*.

### 7.3.3 Cytolysin

Cytolysin (Cyl) or  $\beta$ -haemolysin/bacteriocin is an enterococcal virulence factor which is a cellular toxin that increases virulence in animal models (Ike et al., 1984; Jett et al., 1992, 1994; Chow et al., 1993; Gilmore et al., 1994). In Japan, the hemolytic phenotype was found in 60 % of clinical strains involved in parenteral infection in comparison to only 17 % of isolates from the feces of healthy individuals (Ike et al., 1987). A study in the United States showed similar trends in *E. faecalis* strains isolated from blood (Huycke et al., 1991). However, a study in Germany revealed haemolytic activity in only 16 % *E. faecalis* strains isolated from blood (Elsner et al., 2000). The production of Cyl may evade the host immune response by destroying cells of the immune system such as PMNs and macrophages (Miyazaki et al., 1993). Enterococci with  $\beta$ -hemolytic activity caused a fivefold increased risk of death in patients within 3 weeks of bacteremia in comparison to non-  $\beta$ -hemolytic strains. The production of cytolysin is considered to be a major risk factor associated with pathogenic enterococci (Huycke et al., 1991).

### 7.3.4 Enterococcus surface protein from *E. faecalis* (*Esp<sub>fs</sub>*) and *E. faecium* (*Esp<sub>fm</sub>*)

Shankar and co-workers (1999) were the first to describe an extracellular surface protein (Esp) in a clinical strain of *E. faecalis* MMH594. The Esp has a molecular mass of 202 kDa and is capable of encoding a primary translation product of 1873 amino acids (Foulquié Moreno et al., 2006). Enterococcus surface proteins are produced by *E. faecalis* (*Esp<sub>fs</sub>*) and *E. faecium* (*Esp<sub>fm</sub>*) and their genes are chromosomally encoded (Franz & Holzapfel, 2004). The incidence of *Esp<sub>fs</sub>* is higher among clinical strains of *E. faecalis* than strains isolated from healthy individuals and can play a role in pathogenicity (Franz & Holzapfel, 2004). *E. faecium* *Esp<sub>fm</sub>* was highly conserved in infection-derived isolates and environmental isolates, although absent in food and commensal



isolates (Eaton & Gasson, 2001). *Enterococcus* surface proteins play a role in adhesion as their presence increases cell hydrophobicity, adhesion to abiotic surfaces and biofilm formation *in vitro* (Toledo-Arana et al., 2001). The C-terminal regions of Esp contain a membrane-spanning hydrophobic domain and a cell wall anchor motif involved in anchoring the protein to the bacterial surface (Franz & Holzapfel, 2004). *E. faecalis* Esp<sub>fs</sub> exhibits characteristics of surface protein receptors, designated microbial surface components, recognizing adhesive matrix molecules (MSCRAMMs) that mediate binding to extracellular matrix proteins. Colonization of host tissue by direct ligand-binding activity to the extracellular matrix in the human host is promoted by Esp<sub>fs</sub> (Toledo-Arana et al., 2001). *Enterococcus* surface proteins also play a role in evasion of the host's immune response as the structures of both Esp's are similar to that of MSCRAMMs (Eaton & Gasson, 2002; Hucke et al., 1991; Miyazaki et al., 1993). Both proteins are similar in sequence and global organization and contain a signal sequence followed by an N-terminal region and a core region that consists of repeat units (Eaton & Gasson, 2002; Shankar et al., 1999). The proteins and genes share 89 % identity (Eaton & Gasson, 2002).

#### 7.3.5 Adhesion to collagen from *E. faecalis* (Ace) and *E. faecium* (Acm)

The proteins Ace and Acm contain an N-terminal signal sequence, followed by a collagen-binding A domain, a B region consisting of repeat units and a cell wall domain with a characteristic LPKTS motif. The LPKTS motif is a potential target for hydrophobic residues, namely sortase that may span the membrane. Ace and Acm share 47 % amino acid sequence similarity (Nallapareddy et al., 2003). These proteins might be involved in evasion of the immune response by mechanisms similar to Esp. Ace binds to types I and IV collagen and laminin and plays an important role in enterococci pathogenesis (Nallapareddy et al., 2000a; Nallapareddy et al., 2000b). This occurs especially during translocation and when the extracellular matrix proteins are exposed to damaged intestinal epidermal layer. A similar mechanism exists for evading the immune system and adhesion. The collagen-binding protein from *E. faecium*, Acm, binds to collagen types I and IV which is regarded as a virulence property (Nallapareddy et al., 2003).

#### 7.3.6 *Enterococcus endocarditis* antigen from *E. faecalis* (EfaA<sub>fs</sub>) or *E. faecium* (EfaA<sub>fm</sub>)

*E. faecalis* and *E. faecium* produce adhesion-like endocarditis antigens (EfaA<sub>fs</sub> and EfaA<sub>fm</sub>, respectively) which are considered to be potential virulence determinants (Franz & Holzapfel, 2004). Growth of *E. faecalis* in serum induced the expression of EfaA (Lowe et al., 1995).

EfaA<sub>fs</sub> plays a role in adhesion in endocarditis and only the *efaA<sub>fs</sub>* gene increases pathogenicity in animal models (Singh et al., 1998). The *efa* operon consist of three genes (*efaC*, *B* and *A*) with homology to ABC-type metal ion transport systems (Low et al., 2003). The gene *efaC* encodes an ATP-binding protein, *efaB* a hydrophobic transmembrane protein, and *efaA* a solute-binding protein receptor for the ABC transport complex (Low et al., 2003). A manganese-regulated operon, EfaCBA, functions as a high-affinity manganese permease in *E. faecalis*. These antigens play a role in the infection of human tissues, where the availability of Mn<sup>2+</sup> is very low (Low et al., 2003).

### 7.3.7 Gelatinase (Gel)

Gelatinase is an extracellular metallo-endopeptidase involved in the hydrolysis of a variety of substrates, including gelatin, collagen, haemoglobin, and other bioactive peptides (Su et al., 1991). Enterococci isolated from patients with endocarditis and other nosocomial infections produced protease (Gold et al., 1975). The gene for gelatinase, *gelE*, is located in an operon together with *sprE* encoding a serine protease (Qin et al., 2000). GelE is a virulence factor of enterococci especially for peritonitis in mice and is commonly produced by nosocomial, fecal, and clinical enterococcal isolates (Singh et al., 1998). Sixty-three percent of enterococci isolates and 54 % isolates from patients with endocarditis and other nosocomial infections were protease-producing *E. faecalis* strains (Coque et al., 1995; Kühnen et al., 1988). GelE cleaves fibrin. The protease is secreted, damages host tissue and allows bacterial migration and spreading thereof (Franz & Holzapfel, 2004). Enterococci, involved in blood infections and vegetations formed during endocarditis, are coated with polymerized fibrin (Waters et al., 2003). The fibrin layer covering the bacteria is degraded after expression of *gelE* which leads to further dissemination of the organism. GelE affects a variety of important housekeeping functions as it clears the bacterial cell surface of misfolded proteins and leads to activation of an autolysin (Waters et al., 2003). The function of this muramidase-1 autolysin is to reduce chain length (Waters et al., 2003). Sex pheromones and their inhibitors are also degraded by GelE and also it plays a crucial role in dissemination of the organism in a high-cell-density environment (Waters et al., 2003). Dissemination of fibrin leads to autolysin activation and reduction in chain length. Degradation of sex pheromones decreases aggregation of enterococci which increases the potential for dissemination (Waters et al., 2003). Another GelE-associated enterococcal virulence factor is the degradation of antimicrobial peptides which are part of the innate immune system (Schmidtchen et al., 2002). The antibacterial peptide LL-37, isolated from epithelial cells,

neutrophils, and sub-populations of lymphocytes and monocytes, was inactivated by the supernatant from a gelatinase-expressing *E. faecalis* strain (Schmidtchen et al., 2002). The presence of Gel is very high among food isolates of *E. faecalis* (Eaton & Gasson, 2001; Franz et al., 2001).

#### 7.3.8 Hyaluronidase

Hyaluronidase degrades hyaluronic acid, a major component of the extracellular matrix of the endothelium. The hyaluronidase gene (*hyl<sub>Efm</sub>*) from a clinical *E. faecium* strain is 1659 bp long and encodes a putative protein of 533 amino acids with a molecular weight of 65, 051 Da (Franz & Holzapfel, 2004). The production of hyaluronidase may play a role in enterococcal pathogenesis. The genes *esp<sub>fm</sub>* and *hyl<sub>Efm</sub>* were found in vancomycin-resistant *E. faecium* isolates from patients in the United States (Rice et al., 2003). Specific *E. faecium* strains may be enriched in determinants that render them more likely to cause clinical infections (Rice et al., 2003).

#### 7.3.9 Capsule

The capsule that surrounds enterococci is a polysaccharide consisting of a repeat structure of kojibiose linked 1,2 to glycerolphosphate (Huebner et al., 1999). The capsule of enterococcal strains protects the bacterial cell from the host's defense mechanisms (Hancock & Gilmore, 2002). This was evident in a murine cutaneous infection model where a mutant was more readily cleared from a resulting abscess than the capsular polysaccharide-producing strains.

#### 7.3.10 Other virulence determinants

An *E. faecium* strain was involved in a recent outbreak of sepsis in humans and pigs (Lu et al., 2002). The investigators believe that this particular strain may harbor gene(s) encoding toxin(s) similar to streptococcal pyrogenic exotoxins (*spe*) and should be studied in future (Lu et al., 2002).

All of the above contributes to the controversial safety issue of enterococci in foods and probiotic supplements. However, they are associated with the human environment and gastro-intestinal tract and the chance of causing diseases in healthy humans is very limited. Conditions such as disturbed physiological condition, underlying disease and immunosuppression are the major causes of enterococcus infection (Franz & Holzapfel, 2004).

## 8. Health benefits

### 8.1 Immune stimulation

The benefits of consuming probiotics are well documented. However, until recently the mechanisms by which probiotics exert their effects have been largely unknown (Heyman, 2000; Parvez et al., 2006). Results obtained from *in vitro* systems, animal models and human trials suggest that probiotics enhance both specific and non-specific immune responses (Parvez et al., 2006). Stimulation of the host's nonspecific and specific humoral immune responses to potentially harmful antigens has been documented for strains of *Bifidobacteria bifidum*, *Bifidobacteria breve* and *Lactobacillus rhamnosus* GG. These effects are mediated through activating macrophages, increasing levels of cytokines, increasing natural killer cell activity, altering the balance of Th1 and Th2 and/or increasing levels of immunoglobulins (Fang et al., 2000; Parvez et al., 2006). The direct effects of probiotics in the gastro-intestinal tract include up-regulation of immunoglobulins such as IgA, down regulation of inflammatory cytokines and enhancement of gut barrier functions. Microbes and antigens interact with components of the immune system along the mucosal surface of the gastro-intestinal tract. Most importantly, these positive effects are exerted on the immune system without eliciting a harmful inflammatory response (Kopp-Hoolihan, 2001).

The first line of host defense is directed toward the exclusion of antigens and regulation of antigen-specific immune responses. As a result the gastro-intestinal barrier controls antigen transport and generation of immunologic phenomena in the gut. These regulatory events constituting the intestinal immune response take place in organized lymph epithelial tissue and secretory sites. The organized lymphoid tissue is composed of Peyer's patches, lymphocytes and plasma cells that are distributed throughout the lamina propria. Intra-epithelial lymphocytes are located above the basal lamina in the intestinal epithelium. The lymphocyte maturation cycle involves antigen transport across Peyer's patches and the presentation of antigens to T lymphocytes which proliferate and induce B cell response (Isolauri et al., 2001). Although blood-borne and tissue immunity has a predominance of immunoglobulin (Ig) G antibodies, compared with IgA and IgM, IgA antibody production is abundant at mucosal surfaces. IgA exists in the monomeric form in circulating human blood, whereas the dimeric form is expressed in secretions of mucosal surfaces including the intestine, saliva and tears (Pfeifer & Rosat, 1999). These secretory IgA antibodies in the gut form part of the common mucosal immune system, including the respiratory tract and lacrimal, salivary and mammary glands. The common

mucosal immune system refers to the generalization of immune responses elicited at mucosal inductive sites and is accomplished by the migration of lymphocytes from the inductive site, via the mesenteric lymph nodes and circulation, to remote mucosal sites (Svennerholm & Quiding-Järbrink, 2003). Probiotics activate the dendritic cells within the Peyer's patches. Dendritic cells are the most potent cells to initiate a primary T-cell-dependent immune response (Clancy, 2003). The balance of the different T helper (Th) subsets is particularly important in mucosal immunity (Dugas et al., 1999). After priming, memory B and T cells migrate to effector sites. This is followed by active proliferation, local induction of certain cytokines and production of secretory antibodies (IgA). Upon antigen exposure, immune cells respond with the release of a host of cytokines that then direct the subsequent immune responses. One of the major mechanisms by which the gut associated lymphoid tissue (GALT) maintains homeostasis is via local cytokine regulation, particularly TGF- $\beta$ -associated low-dose tolerance immunity (Dugas et al., 1999).

IgA is resistant to intraluminal proteolysis and does not activate complement or inflammatory responses, which makes secretory IgA ideal for protecting mucosal surfaces. Results obtained from experimental animal models suggested that the capacity to generate IgA-producing cells is initiated with the establishment of the gut microflora and that the secondary lymphoid organs, i.e. the spleen and lymph nodes, are poorly developed in germfree animals because of the lack of antigenic stimulation (Jiang et al., 2004). The role of intestinal microflora in oral tolerance induction (i.e. the unresponsiveness to nonpathogenic antigens encountered at the mucosal surface) to the IgE response was investigated in germfree mice. In contrast with control mice, germfree animals maintained their tendency to systemic immune response, e.g. the production of IgE antibodies, after oral administration of ovalbumin. The aberrant IgE response in germfree mice could be corrected by reconstitution of the microflora at the neonatal stage but not later (Jiang et al., 2004). These results suggest that the gut microflora direct the regulation of systemic and local immune responsiveness by affecting the development of GALT at an early age. In humans, colonization was associated with the maturation of humoral immune mechanisms particularly of circulating IgA and IgM secreting cells. The specific IgA response could contribute to the preventative potential of probiotics. When antigens penetrate through the oral route, a secretory immune response is obtained which is mediated by secretory IgA or IgM (Jiang et al., 2004). The mucus secreted by epithelial cells is the first layer of mucosal defense and consists of a combination of IgA, antimicrobial peptides, and a complex mixture of glycosylated proteins coded by several genes. IgA is secreted by B lymphocytes (plasma cells) in the lamina

propria of the mucosa and is directed to specific antigens present in the lumen of the gastro-intestinal tract and even to antigens from commensal bacteria (Saavedra & Frank, 2002). The host makes a humoral mucosal response to all gut bacterial species characterized by an increase in IgA production in the gastro-intestinal tract. Each bacterial species has a characteristic stimulation marked by a particular steady state level of gastro-intestinal tract IgA production and a ratio of specific IgA to general IgA production. The best documented clinical application of probiotics is in the treatment of acute diarrhoea and as adjunct therapy in gut-related inflammatory conditions (Isolauri, 2001; Kaur et al., 2002).

Diarrhoea results from disequilibrium in water movement across the intestinal epithelium. Multiple factors are responsible for regulating water and electrolyte absorption and secretion and therefore the beneficial effects of probiotics involve many different mechanisms. *Saccharomyces boulardii* and *L. rhamnosus* GG increase secretory IgA levels in the gastro-intestinal tract (Parvez et al., 2006). *L. rhamnosus* GG is commonly used in commercial preparations and modulates intestinal immunity by increasing the levels of IgA and other immunoglobulin secreting cells in the intestinal mucosa (Kaur et al., 2002). This strain increased the rotavirus-specific IgA response in children with rotavirus diarrhoea (Fang et al., 2000; Heyman, 2000; Isolauri et al., 2001). The increase in rotavirus-specific IgA was significantly higher with strain GG compared to a combination of *Streptococcus thermophilus* and *Lactobacillus delbruckii* subsp. *bulgaricus*. Consumption of *L. rhamnosus* GG shortened the phase of diarrhoea from an average of 3.5 days to 2.5 days in children being treated for rotavirus infection (Cross, 2002; De Roos & Katan, 2000). Both active and non-active preparations of strain GG reduced the duration of rotavirus diarrhoea, however only active forms increase the level of specific-IgA secreting cells to rotavirus (Fang et al., 2000). Infants that received *L. rhamnosus* GG showed temporary increases in IgG, IgA and IgM levels and cells secreting specific-IgA were present at higher numbers compared to controls thereby providing protection against re-infection (De Roos & Katan, 2000).

Volunteers that ingested *Lactobacillus johnsonii* La1 and milk fermented with bifidobacteria showed a 4-fold increase in specific IgA after ingesting an attenuated strain of *Salmonella typhi*, compared to a control group (De Roos & Katan, 2000; Heyman, 2000; Pfeifer & Rosat, 1999). When pre-feeding of probiotics was initiated 21 days before vaccination there was a significant increase in the pathogen-specific IgA response, while 7 days pre-feeding induced a non-

significant trend toward an increase in the same parameter (Cross, 2002). The specific IgA titer to *S. typhi* Ty21a in human volunteers was increased by fermented milk containing *L. johnsonii* La1 and bifidobacteria (Fang et al., 2000). *L. rhamnosus* GG stimulated IgA antigen secreting cell responses against *S. typhi* Ty21a in greater numbers compared to *Lactobacillus lactis* and a placebo control. *L. rhamnosus* GG increases antigen transfer across Peyer's patches to underlying lymphoid cells. This may explain the different responses observed (Kaur et al., 2002). In two groups of adult volunteers that received typhoid vaccine, the antibody titers were significantly higher in the group that received *L. rhamnosus* GG (Young & Huffman, 2003). Strain GG also increased rotavirus-specific IgM secreting cells in infants that received an oral rotavirus vaccine (Cross, 2002; Heyman, 2000). Yoghurt supplemented with *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Bifidobacterium infantis* enhanced mucosal and systemic IgA responses to cholera toxin immunogen in mice (Kaur et al., 2002). Feeding mice with *L. casei* Shirota prior to influenza virus challenge also significantly increased protection of the upper respiratory tract (Cross, 2002). *L. casei* Shirota and *E. coli* O157:H7 fed to infant rabbits exhibited a lower incidence of severe diarrhoea and lower levels of Shiga toxins 1 and 2 were present in the gastro-intestinal tract compared to infant rabbits fed only with *E. coli* O157:H7. Anti-*E. coli* and anti-toxin IgA levels were higher in the gastro-intestinal tract tissue of the animals that were fed probiotics compared to control animals. When mice were fed a combination of *L. acidophilus*/*L. casei* an increase in survival against pathogens was observed and both serum and gastro-intestinal tract mucosal anti-Salmonella antibody titers were elevated (Cross, 2002). These results were also observed for anti-Shigella antibodies when mice were challenged with *Shigella sonnei*. These results indicate that some LAB have the ability to persist in the intestinal tract and act as adjuvants to the humoral immune response (Cross, 2002).

Macrophages, neutrophils, eosinophils, NK cells, epithelial cells and M cells monitor the sites of pathogen entry and coordinate early defense by the innate gastro-intestinal system (Dugas et al., 1999). Phagocytosis is responsible for early activation of the inflammatory response before antibody production. Phagocytic activity results in the further recruitment of immunocompetent cells and the generation of inflammatory response. Therefore, an increase in the number of activated phagocytes is indicative of non-specific immune stimulation and signals the strengthening of general defence mechanisms (Pfeifer & Rosat, 1999). Oral introduction of *Lactobacillus casei* and *Lactobacillus bulgaricus* activates the production of macrophages and administration of *L. casei* and *Lactobacillus acidophilus* activates phagocytosis in mice (Isolaure



et al., 2001). Enhanced phagocytosis was also reported in humans by *L. johnsonii* La1. Probiotic bacteria modulate phagocytosis differently in healthy and allergic subjects. An immunostimulatory effect was observed in healthy persons, whereas in allergic persons, down-regulation of the inflammatory response was detected. In a study of the immunomodulation following the consumption of milks fermented with *B. bifidum* or *L. johnsonii* LA1, human blood samples showed an increased phagocytosis of *E. coli* *in vitro* (Heyman, 2000). When *L. rhamnosus* GG was given to volunteers the number of white blood cells with phagocytic activity doubled (De Roos & Katan, 2000). *L. acidophilus* and *B. bifidum* had little effect on immunity in elderly volunteers, only B lymphocytes increased significantly. This suggests that host characteristics also contribute to the different effects exerted by probiotics. In a study to determine the effect of consuming different doses and different strains of LAB on immune indices, one group of volunteers consumed  $10^{10}$  cfu *B. bifidum* Bb12 and a second group received  $7 \times 10^{10}$  cfu *L. johnsonii* La1 daily for 3 weeks (Pfeifer & Rosat, 1999). Two other groups each consumed either  $10^9$  cfu or  $10^8$  cfu of strain La1 daily. The increase in leucocyte phagocytic activity was significant in both groups after the ingestion of LAB, but was more evident in the group ingesting bifidobacterium. Overall, phagocytic activity decreased 6 weeks after probiotic ingestion was stopped, but the group that consumed La1 retained the highest activity. The respiratory burst and phagocytic activity were significantly enhanced in volunteers who consumed  $10^9$  cfu La1, but not in those who consumed a lower dose of  $10^8$  cfu. In animals, LAB also exhibit immune stimulating capacity. Different strains of *Lactobacillus* and *Streptococcus thermophilus* were capable of stimulating non-specific (macrophages) and specific (lymphocytes B and T) immunity in mice (Heyman, 2000). *Lactobacillus acidophilus* UFV-H2b20 stimulates a non-specific immune response in germ-free Swiss mice as indicated by stimulation of the host mononuclear phagocytic activity (Kaur et al., 2002). There was a two-fold increase in the number of Kupffer cells, responsible for the clearance of circulating bacteria. Enhanced phagocytosis was substantiated in humans by *L. johnsonii* La1 and *L. rhamnosus* GG (Isolauri, 2001). In addition to enhanced pathogen-specific antibody production, strains of *Lactobacillus* and *Bifidobacterium* spp. have also resulted in an increase in non-specific *in vivo* phagocytic activity or peritoneal macrophages and blood-borne neutrophils following pathogenic challenge (Cross, 2002). This suggests that enhanced cell mediated immunity may also contribute to increased protection. Rats fed with *L. casei* Shirota prior to oral challenge with *Listeria monocytogenes* showed reduced pathogen burdens in several excised GI tract tissues and lower pathogen translocation to the spleen and liver. The probiotic-fed rats showed an increased level



of *in vivo* lymphocyte sensitization to microbial antigens. Intestinal microorganisms could down-regulate the allergic inflammation by counter-balancing T-helper cell Type-2 responses and by enhancing antigen exclusion through induction of an IgA response (Kaur et al., 2002). *L. rhamnosus* GG and *Propionibacterium freudenreichii* spp. *shermanii* JS showed an immunomodulatory effect on the proliferate activity of murine B and T lymphocytes. The oral administration of *Lactobacillus casei* CRL431 in mice stimulated type 1 helper T (Th1) cells, activated the cellular immune system and inhibited IgE production. The cell wall of *Lactobacillus casei* CRL431 presents lectin-like surface molecules which stimulate the immune system. Humans consuming fermented milk containing *L. casei* Shirota daily for 3 weeks showed increased levels of natural killer (NK) cell activity (Commane et al., 2005). Natural killer activity returned to levels comparable with the controls 3 weeks after the feeding period had ended. An increase in NK activity was also observed when *L. casei* strain Shirota was fed to mice.

Specific strains of the normal, healthy gut microflora promote gut barrier functions, give maturational signals for the gut associated lymphoid tissues and balance the generation of pro- and anti-inflammatory cytokines thereby creating healthy interactions between the host and microbes in the gut that are needed to keep inflammatory responses regulated but concomitantly readily primed (Isolauri, 2001). Even in the absence of inflammatory stimuli from the environment, the healthy and mature intestine is in a proinflammatory state, provoking many differentiated and activated lymphocytes that generate proinflammatory cytokines, a state called controlled inflammation (Isolauri, 2001). When the gastro-intestinal tract becomes inflamed it becomes permeable and serves as a link between inflammatory disease in the gastro-intestinal tract and other disorders such as arthritis (Parvez et al., 2006). An inflammatory immune response produces cytokine-activated monocytes and macrophages which release cytotoxic molecules and there is an increasing appreciation of the role of cytokines in regulating inflammatory responses at a local and systemic level. Several strains of live LAB have been shown to induce *in vitro* the release of the proinflammatory cytokines TNF- $\alpha$  and IL-6, reflecting stimulation of nonspecific immunity (Isolauri et al., 2001). Pro-inflammatory cytokines play a pivotal, yet paradoxical role, in inflammation. Experiments in cytokine transgenic knockout mice show that a harmless immune response to commensal gut microflora becomes a harmful inflammatory state in the absence of IL-10, TGF- $\beta$  and IL-2 and can lead to chronic gastro-intestinal tract inflammatory diseases (Dugas et al., 1999). This indicates that inflammation is

induced by an unbalanced local or systemic cytokine environment. Probiotics may have an indirect impact on immunity by modulating inflammatory mediators such as cytokines. The ingestion of probiotic bacteria can potentially stabilize the immunological barrier in the gut mucosa by reducing the generation of local proinflammatory cytokines. The regulatory role of specific strains of the gut microflora was shown previously by a suppressive effect of immune responses to dietary antigens in allergic individuals partly attributable to enhanced production of anti-inflammatory cytokines IL-10 and TGF- $\beta$ , whereas the capacity to stimulate nonspecific immune responses was retained. Recently probiotics modulated the host's immune responses to foreign antigens with a potential to dampen hypersensitivity reactions. Unheated and heat-treated homogenates were prepared from probiotic strains, including *L. rhamnosus* GG, *B. lactis*, *L. acidophilus*, *L. delbruckii subsp. bulgaricus* and *S. thermophilus*. The phytohemagglutinin-induced proliferation of mononuclear cells was suppressed in these homogenates compared with controls with no homogenates, indicating that probiotic bacteria possess heat-stable, antiproliferative components, which could be therapeutically exploited in inflammatory conditions. Probiotics play a role in down regulating inflammation associated with hypersensitivity reactions in patients with atopic eczema and food allergies (Kaur et al., 2002; Parvez et al., 2006; Young & Huffman, 2003). Probiotics also showed up-regulation of anti-inflammatory cytokines, such as IL-10, in atopic children. In this way probiotics play a role both in immunostimulation in healthy individuals and down-regulation of immunoinflammatory responses in hypersensitive individuals.

*L. bulgaricus* 100158 and *S. thermophilus* 001158 fed to rats increased lymphocyte proliferation in the spleen, peripheral blood and Peyer's patches and elevated IFN- $\gamma$  production in the Peyer's patches and spleen (Commane et al., 2005). Splenocytes cultured *in vitro* displayed an increase in the inflammatory immune response associated with IL-12 when given an oral application of *L. casei* strain Shirota. Macrophage cell lines and murine cultures composed of peritoneal, spleen and Peyer's patch cells were used to examine the effect of heat-killed cells, cell walls and cytoplasmic extracts of *Bifidobacterium*, *Lactobacillus acidophilus*, *L. bulgaricus*, *L. casei*, *L. gasseri*, *L. helveticus*, *L. reuteri* and *Streptococcus thermophilus* effects on cell proliferation and cytokine and nitric oxide (NO) production (Kaur et al., 2002). Both the cell wall and cytoplasmic fractions were able to stimulate cloned macrophages to produce significant amounts of TNF- $\alpha$ , IL-6 and NO. Increased IFN- $\alpha$  activity was observed in virally-challenged peripheral blood samples from humans that consumed *L. brevis* subsp. *coagulans* (Commane et al., 2005). A

decrease in TNF- $\alpha$  production was observed in human ileal specimens from Crohn's disease patients treated *ex vivo* with *L. casei* DN114001 and *L. bulgaricus* LB10. TNF- $\alpha$  changes were not observed in non-inflamed mucosa indicating a down-regulation of Th1-like cytokines associated inflammation by probiotics. *L. rhamnosus* GG significantly decreased the concentration of TNF- $\alpha$  in fecal samples from infants with dermatitis and cow milk allergy compared to a placebo control group (De Roos & Katan, 2000; Dugas et al., 1999; Isolauri et al., 2001). Paradoxically, ingestion of lactobacilli in fermented milk products or as live attenuated bacteria was shown to increase the IFN- $\gamma$  production by peripheral blood mononuclear cells. IFN- $\gamma$  can promote the uptake of antigens in Peyer's patches where specific IgA-committed cells are generated. An increase in systemic and mucosal IgA response to dietary antigens was shown after oral administration of lactobacilli. Similarly, feeding an extensively hydrolysed whey formula supplemented with *L. rhamnosus* GG improved the clinical score of atopic dermatitis and decreased the intestinal excretion of  $\alpha$ 1-antitrypsin and TNF- $\alpha$  compared with children fed the extensively hydrolyzed formula alone (Heyman, 2000). A mixture of bovine caseins, hydrolyzed with *L. rhamnosus* GG, derive enzymes that may induce a suppression of lymphocyte proliferation and a down regulation of IL-4 production *in vitro*. Bacterial VSL#3 DNA has shown to down regulate proinflammatory cytokine secretion by attenuation of the nuclear factor- $\kappa$ B pathway in intestinal an epithelial cell chemically induced colitis model in mice (Marteau & Shanahan, 2003). The beneficial effect of the probiotic mixture VSL#3 on the immune system was derived from its DNA as VSL#3 genomic unmethylated DNA was effective whereas VSL#3 methylated DNA and calf thymus DNA were ineffective. The stimulation of dendritic cells by CpG DNA is associated with the production of T-helper type 1 (Th1)-like cytokines such as IL-12. Immune responses are different in mice given oral preparations of *B. breve* YIT4064 compared to mice given *L. casei*. *Bifidobacterium* sp. raised a Th-2 type response that is characterized by increased IgG activity against oral infection with an influenza strain. *Lactobacillus* sp. induced the expression of the type-1 helper T-cell associated cytokines IFN- $\gamma$  and IL-2 (Commane et al., 2005).

The host is able to distinguish signals from pathogens and commensals by pattern recognition receptors or Toll-like receptors (TLRs) which are differentially expressed between immune cells and by intestinal epithelial cells (Marteau & Shanahan, 2003). TLRs are transmembrane proteins that respond to different microbial antigens and are able to discriminate between different microbes by detecting several features of a microorganism simultaneously (Clancy, 2003). Ligand-specific binding promotes interaction of the cytoplasmic domain with adaptor proteins

followed by the recruitment of kinases and activation of downstream target effector systems, including MAPK as well as the NF-  $\kappa$ B transcriptional system. The transcription factor nuclear factor NF-  $\kappa$ B transcriptionally regulates the expression of TLRs as well as a wide range of chemoattractant and inflammatory cytokines (Ruiz et al., 2005).

Regular consumption of probiotics allows their interaction with immune components of the gastro-intestinal tract. This interaction results in the stimulation and modulation of the immune system of the host. Most probiotic effects on the immune system are a result of regulating the balance between pro- and anti-inflammatory cytokine production. The most well documented immunological effects are the modulation of the inflammatory response and use as an adjuvant. However, the way in which the immune system is modulated depends on a number of factors including the immunologic state of the host and the specific strain/strains of probiotics used. Different microorganisms produce different effects on the immune system. Cytokine secretion patterns and the specific immunomodulatory effect of the probiotic is a strain-dependent characteristic and therefore the effects of each strain need to be evaluated individually and specific strains can be used in the treatment of specific diseases.

## 8.2 Infectious diseases

One of the biggest problems yet to solve in the 21<sup>st</sup> century is infectious diseases in humans. Pathogenic microorganisms which cause these diseases and subsequently lead to death in developing countries include *Shigella*, *Vibrio cholera*, pathogenic *Escherichia coli*, *Campylobacter*, and rotavirus. Even in developed countries many cases of diarrhoea in children as well as food borne infection still occur. Probiotic bacteria have shown to be effective in the prevention of infections such as acute diarrhoea, *Helicobacter pylori*-infections, necrotizing enterocolitis, *Campylobacter*-induced enteritis and infections in the urinary tract in double-blind placebo-controlled randomized studies (Alvarez-Olmos & Oberhelman, 2001; Bengmark, 2003; Gill, 2003; Reid et al., 2003).

Rotaviral diarrhoea is identified by vomiting and subsequent rapid watery diarrhoea and occurs mainly in infants aged 6 months to 2 years. The impaired mucosa leads to unbalanced microflora and subsequently osmotic diarrhoea and diarrhoea caused by bacterial overgrowth (Isolauri et al., 1994). Clinical trials showed that probiotics may treat rotaviral infection by reducing diarrhoea and frequency and that efficacy improves with increased dosages (Kaila et al., 1992; Szajewska

& Mrukowicz, 2001; Van Niel et al., 2002). In a multi-centre study in Europe, a significantly lower duration of diarrhoea in 1 to 3 month old neonates was observed compared to the placebo group (Guandalini et al., 2000). Children aged 6 to 36 months, of whom 75 % were infected with rotavirus, ingested *L. reuteri* SD 2222. Watery diarrhoea was shortened to five days (Shornikova et al., 1997). Two other placebo-controlled studies with *L. rhamnosus* GG showed reduction of nosocomial rotavirus gastroenteritis and protective effects against bacterial and viral pathogens in undernourished children (Oberhelman et al., 1999; Szajewska et al., 2001).

Antibiotics such as clindamycin, cephalosporin and penicillin might induce diarrhoea due to disturbance of the normal microflora and the increased growth of endogenous *C. difficile* in the intestine (Nomoto, 2005; Sullivan & Nord, 2002). The probiotic *S. boulardii* produces a proteolytic enzyme which digests toxin A or B of *C. difficile* and receptors of these toxins in the epithelium of the intestine. In two clinical trials diarrhoea decreased by 11 % and 18 % respectively in children on antibiotic therapy treated with *L. rhamnosus* GG (Arvola et al., 1999; Vanderhoof et al., 1999). Results of other double-blind placebo-controlled studies on antibiotic-induced diarrhoea also showed significant effects of probiotics (D'Souza et al., 2002). Traveler's diarrhoea caused mainly by *E. coli*, shigella and salmonellae occurs in 10 – 60 % of people traveling from developed countries to subtropical and tropical zones (DuPont & Ericsson, 1993). Diarrhoea was significantly decreased with the ingestion of *L. rhamnosus* GG by travelers to one region (Oksanen et al., 1990). In other studies there were no significant effects with probiotics and more studies are needed to confirm the effect of probiotics on traveler's diarrhoea (Katelaris et al., 1995; Oksanen et al., 1990).

Necrotizing enterocolitis, occurring in neonates, shows increased intestinal bacterial flora such as *Enterococcus*, *E. coli*, *Staphylococcus*, and *Clostridium perfringens* which aggravate the symptoms of this disease (Nomoto, 2005). Probiotic administration showed a 60 % decrease of necrotizing colitis (Hoyos, 1999). However, not a significant difference was found in other studies (Dani et al., 2002; Millar et al., 1993).

Probiotics together with prebiotics (non-digestible food ingredients) have been used in the prevention of infectious diseases after surgery in the gastro-intestinal tract. Administration of *L. plantarum* 299 decreased the incidence of postoperative infectious disease from 48 % to 13 % and improved recovery of immunity (Rayes et al., 2002a). The development of sepsis after

surgery in the abdominal cavity was also significantly decreased (Rayes et al., 2002b). Kanazawa and co-workers (2005) concluded that postoperative enteral nutrition supplemented with *L. casei* Shirota strain, *B. breve* Yakult strain and galactooligosaccharides improved the intestinal microflora and the concentration of intestinal organic acid was normalized in patients. Infectious complications were significantly decreased and the stay in hospital and need for antibiotic administration were shortened. Bacterial translocation (BT) is defined as ‘the passage of viable enteric bacteria from the intestinal lumen through the epithelial mucosa into the lamina propria and then into mesenteric lymph nodes (MLNs) and possibly other organs (Wiest & Rath, 2003). Probiotics and their production of organic acid might improve the resistance of the host against the factors causing BT in infectious diseases after operation of digestive organs (Nomoto, 2005).

The microaerophilic Gram-negative *Helicobacter pylori* is strongly associated with gastritis, peptic ulcer and possibly gastric cancer. Clinical studies with *L. acidophilus* (viable and heat-killed) and *L. johnsonii* La1 significantly decreased the symptoms of *H. pylori* in patients. The mechanism of the probiotic bacteria is not clear. However, it was hypothesized that the killed *L. acidophilus* bacteria improved the host immune system and prevented adsorption of *H. pylori* to glycolipid receptors (Canducci et al., 2000; Felley et al., 2001; Michetti et al., 1999; Sakamoto et al., 2001). The infection-preventing effects of probiotics possibly include the activation of the innate immune system and/or inhibiting the adhesion of pathogenic bacteria to the epithelium by competitive antagonism (Nomoto, 2002).

High numbers of *Lactobacillus* bacteria form part of the normal bacterial flora in the vagina and ensure a reduced risk of bacterial vaginosis and urinary tract infections (Nomoto, 2005; Reid & Burton, 2002). Lactobacilli inhibited the growth and attachment of uropathogenic *E. coli* *in vitro* (McGroarty & Reid, 1988; Reid et al., 1987; Velraeds et al., 1998). *L. rhamnosus* GR-1, *L. fermentum* B-54 and *Lactobacillus* strains with high adhesion and hydrogen peroxide production were weekly administered as a vaginal suppository (Reid & Bruce, 1995; 2001; Reid et al. 1987; 1995a; 1995b). Urinary tract infection was significantly decreased. Orally administered *L. rhamnosus* GR-1 and *L. fermentum* RC-14 decreased *E. coli* and fungi in the vagina. Future studies should include the inhibition of STBs by probiotics taken orally or as vaginal suppositories (Reid et al., 2003; Sewankambo et al., 1997). A proposed mechanism is the inducing of an immune response via the urethra or vagina which is functional in the bladder (Reid & Burton, 2002). Probiotic colonization in the vagina might prevent infection by



competition for nutrients and mannose and hydrophobic adhesion to receptors (Braun, 1999; Masuoka & Hazen, 1999) and possibly bacteriocin production with fungistatic activity (Okkers et al., 1999).

### 8.3 Prevention and treatment of inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group term for the intestinal disorders Crohn's disease and ulcerative colitis caused by three interactive pathogenic factors, namely genetic susceptibility, environmental factors and immune dysregulation (Shanahan, 2001; 2002; 2003). Therapeutic and preventative strategies are implemented to target the immune response (Shanahan, 2003). However, these immunomodulatory drugs are expensive, toxic and not always effective. A new strategy emerged to improve the beneficial microflora of the host as a way of limiting the development of IBD. However, several clinical studies do not support the use of probiotics in the maintenance therapy of ulcerative colitis (Ishikawa et al., 2000; Kruis et al., 1997; Kruis et al., 2001; Rembacken et al., 1999). Treatment of active ulcerative colitis by probiotics is not performed because of the possibility of sepsis. Future research is therefore needed to determine the effective use of probiotics in maintenance therapy (Tamboli et al., 2003). Chronic pouchitis is defined as 'a non-specific inflammation of the ileal reservoir following the surgical creation of an ileal-anal anastomosis with a pouch reservoir, in the setting of ulcerative colitis. The symptoms include abdominal pain, diarrhoea, urgency, rectal bleeding and possibly fever. The lyophilized bacterial combination, VSL#3, which includes strains of *Lactobacillus* spp., *Bifidobacterium* spp. and one *Streptococcus* sp., reduced the rate of relapse of chronic pouchitis. The therapeutic effect was not maintained after treatment because the strains in VSL#3 did not stay viable in the colon (Gionchetti et al., 1999; 2000a; 2000b; Mimura et al., 2002). The lactobacilli, bifidobacteria and streptococci increased concentrations in the faeces and the tissue levels of IL-10 that might relate to the therapeutic efficacy (Tamboli et al., 2003). Treatment of Crohn's disease with the probiotics Mutaflor® (*E. coli* Nissle strain 1917, serotype 06K5HI), *Saccharomyces boulardii* and *L. rhamnosus* GG showed no significant effect in controlled studies (Guslandi et al., 2000; Malin et al., 1996; Malchow, 1997; Prantera et al., 2002). Future application of probiotics in IBD might include genetically modified (GM) strains. *L. lactis* was used as a vector in an animal model of colitis to deliver active interleukin (IL) 10. The GM treatment showed similar results to other conventional IBD treatments (Steidler et al., 2000). However, the applicability, safety, and economic feasibility for humans should be studied first.

#### 8.4 *Treatment of irritable bowel syndrome (IBS)*

This relapsing chronic disorder is characterized by recurrent abdominal pain and altered bowel function. One fifth of the general population suffers from these clinical symptoms but only a few seek medical therapy (Carlson, 1998; Jones et al., 2000; Olden, 2002; Thompson & Gick, 1996). Irritable bowel syndrome increases health-care costs as it leads to disability, absence from work or school. In the few probiotic studies using *Lactobacillus* spp. and VSL#3 abdominal bloating and gastro-intestinal function were significantly improved (Kim et al., 2003; Kim et al., 2005). Colonic transit was also retarded by VSL#3 (Kim et al., 2005). Mechanisms of the probiotic therapeutic effect include anti-inflammatory effects, modulating of intestinal flora and increased motility. The anti-inflammatory effects summarized in Table 14 show in general a decreased release of TNF- $\alpha$  in ileal tissues, reduction of IL-8 production and an increased IL-10 production. Colonic bacteria which produce gas and short chain fatty acids might be reduced by probiotic bacteria and therefore inhibit propulsive contractions (Kamath et al., 1990). Probiotic bacteria may also reduce the bile acid load in patients with diarrhoea causing secretion of colonic mucin and fluid and colonic contractions (Bampton et al., 2002; Chadwick et al., 1979). This leads to a reduction in the reflex motor responses of the colon and therefore retardation of stool transit (Bazzocchi et al., 2002).

#### 8.5 *Anticarcinogenic activity*

Probiotic intake may prevent colon and bladder cancer (Gibson & Macfarlane, 1994; Morotomi et al., 1990). Researchers believe that microflora in the colon play an important role in the development of colon cancer (Rowland, 1988). Several species of bacteria found in the colon produce carcinogens and tumor promoters from food components that reach the colon. Many of these microorganisms also synthesize enzymes that generate toxic products (Table 15).



Table 14

Summary of literature on effects of bacterial species on inflammatory responses<sup>a</sup>

Species	Cytokines	Present in VSL#3	Reference(s)
<i>L. bulgaricus</i>	↓ TNF- $\alpha$	+	Borrueal et al. (2003)
<i>L. casei</i>	↓ TNF- $\alpha$	+	Borrueal et al. (2003)
<i>L. salivarius</i>	↓ IFN- $\gamma$		McCarthy et al. (2003)
<i>L. reuteri</i>	↓ IL-8	-	Ma et al. (2004)
<i>B. breve</i>	↓ TNF- $\alpha$ ; ↑ IL-10	+	Menard et al. (2004)
<i>S. thermophilus</i>	↓ TNF- $\alpha$	+	Menard et al. (2004)
<i>L. plantarum</i>	↑ IL-10	+	Pathmakanthan et al. (2004)
<i>B. infantis</i>	↓ TNF- $\alpha$ ; ↓ IL-12, ↑ IL-10/ IL-12 ratio	+	McCarthy et al. (2003), O'Mahony et al. (2005)

↑ = increase, ↓ = decrease

Table 15

Toxic products produced by colonic bacteria (obtained from Rastall &amp; Gibson, 2002)

Enzyme	Toxic products
$\beta$ -glycosidase	Plant glycosides, e.g. rutin, cycasin
Azoreductase	Azo compounds, e.g. benzidines
Nitroreductase	Nitro-compounds, e.g. nitrochrysene
$\beta$ -glucuronidase	Biliary glucuronides, e.g. benzidine
IQ hydrazine-dehydrogenase	2-amino-3-methyl-3H-imidazo-4,5-f. quinoline IQ
Nitrate/nitrite reductase	Nitrate, nitrite

Antimutagenic mechanisms proposed for probiotics include the following: Binding of micro-organisms that produce (pro) carcinogens, production of antimutagenic compounds by viable probiotic cells, modulation of procarcinogenic enzymes in the gut, alteration of colonic transit time to remove faecal mutagens more effectively, reduction of the intestinal pH, thereby altering microflora activity and bile solubility and suppression of tumors by enhancing defense immune mechanisms (Hirayama & Rafter, 1999; McIntosh, 1996). Evidence for the possible role of probiotics in the prevention/treatment of colon cancer has come from *in vitro*, animal and human studies (Tables 16 – 20).

Table 16

Bacterial enzyme activities modified by probiotic treatment

Probiotic	Subjects	Treatment	Effect	Reference(s)
<i>L. acidophilus</i> A1, <i>B. bifidum</i> B1, <i>S. lactis</i> , <i>S. cremoris</i>	9 males and females	100 g fermented milk product, 3 times daily/ 3 weeks (wks)	↓ faecal nitroreductase, azoreductase, β-glucuronidase	Marteau et al.(1990)
<i>L. rhamnosus</i> GG (LGG), <i>L. casei</i> strain Shirota	Human volunteers	-	↓ fecal β-glucuronidase, nitroreductase and glycoeholic acid hydrolase activities	Lidbeck et al. (1992)
LGG	Elderly volunteers	20 ml fermented whey preparation for 2 wks	↓ activity of glycolic acid hydrolase, β-glucuronidase, urease	Ling et al. (1992)
LGG	64 female adults	-	↓ activity of nitroreductase, β-glucuronidase, glycolic acid hydrolase, ↓ levels p-cresol in faeces	Ling et al. (1994)

↓ = decrease

Table 17

Anti-genotoxic activity of probiotics *in vitro*

Probiotic	<i>In vitro</i> test	Effect	Reference(s)
<i>L. casei</i>	<i>S. typhimurium</i> Ames assay	Anti-mutagenicity effect against mutagenic nitrosated beef extract.	Renner & Musner (1991)
Omniflora ( <i>B. longum</i> , <i>L. gasseri</i> , <i>E. coli</i> )	<i>S. typhimurium</i> Ames assay	Anti-mutagenicity effect against mutagenic nitrosated beef extract.	Renner & Musner (1991)
LAB	Binding to food borne carcinogens	Binding of 3-amino-1-methyl 5 h pyrido[4,3-b]indole acetate (TrpP2), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,8-demethylimidazo[4,5-f]quinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by LAB	Orrhage et al. (1994)

Table 18

Probiotic effect on promotion phase of carcinogenesis in rats and anti-genotoxic activity of probiotics in rats

Probiotic	Treatment	Effect	Reference(s)
LGG	Exposure to 1,2 dimethyl hydrazine (DMH) which induce tumors	↓ colonic tumors	Goldin et al. (1996)
<i>L. plantarum</i> 299	Interleukin 10 knock-out	Stabilized gut mucosal barrier	Kennedy et al. (2000)
<i>L. brevis</i>	-	↓ take-up of radiolabelled mannitol and improved barrier function	Garcia Lafuente et al. (2001)
<i>L. casei</i> Shirota	Exposure to <i>N</i> -methyl- <i>N</i> -nitro, <i>N</i> -nitroso-guanidine	Inhibited DNA damage in colon	Pool-Zobel et al. (1993)
Different lactobacilli species	Colon carcinogen 1,2-dimethyl hydrazine	Anti-genotoxic effect was species specific.	Pool-Zobel et al. (1996)

Table 19

Stimulation of immune system in humans by probiotic treatment

Probiotic	Subjects	Treatment	Effect	Reference(s)
<i>L. casei</i> preparations	Double blind study, cancer patients	-	Suppressed recurrence of bladder tumors	Aso & Akazan (1992), Aso et al. (1995)
<i>L. casei</i> Shirota	-	-	Inhibition of methyl-cholanthrecene-induced sarcomas	Yokokura (1994)
<i>L. brevis</i> subsp. <i>coagulans</i>	Human volunteers	-	↑ IFN- $\alpha$ activity in peripheral blood	Kishi et al. (1996)
<i>L. casei</i> Shirota (Yakult 400)	Human volunteers	Consumed fermented milk	↑ NK cell activity	Nagao et al. (2000)

Table 20

Stimulation of immune system in mice by probiotic treatment

Probiotic	Treatment	Effect	Reference(s)
<i>L. salivarius</i> UCC118	IL-10 knockout mice	↓ colonic inflammation ↓ tumor development	O'Mahony et al. (2001)
<i>L. casei</i> strain Shirota	Inhibition of methylcholanthracene-induced tumor development	↑ NK cell activity; delayed development of tumors	Takagi et al. (2001)
<i>L. bulgaricus</i> 100158, <i>S. thermophilus</i> 001158	Yoghurt feeding	↑ lymphocyte proliferation in spleen, peripheral blood, Peyer's patches ↑ IFN- $\gamma$ production in Peyer's patches, spleen	Aatouri et al. (2002)
LGG	Implanted bladder tumor cell line	Subcutaneous development was inhibited	Lim et al. (2002)

The anti-carcinogenic effects of probiotics are supported by the above-mentioned studies and the effects involve specific strains/species and a combination of mechanisms. For the use of probiotics as cancer prophylactics in humans, specific strains with the most benefit should be selected and their mechanism(s) identified (Commane et al., 2005).

#### 8.6 Prevention and treatment of allergies

Allergic diseases increased in Western countries during the past few years (Yazdanbakhsh et al., 2002). A possible reason is improved hygienic conditions and therefore a reduced exposure to microbes during childhood (Yazdanbakhsh et al., 2002). Gut microflora differs in children with allergic diseases to healthy children. High numbers of lactobacilli and bifidobacteria are found in the non-allergic and *Staphylococcus aureus* and coliforms are more common in allergic children (Bjorksten et al., 1999). Antiallergenic processes are promoted by the gastro-intestinal microflora and include enhancement of systemic T-helper-1-type responses and IgA production (Gaskins, 1997; Martinez and Holt, 1999), generation of transforming growth factor  $\beta$  (Isolauri et al., 2000; Sanfilippo et al., 2000) which plays an essential role in suppression of T-helper-2-induced allergic inflammation (Hansen et al., 2000) and induction of oral tolerance (Sudo et al., 1997).

The gut microflora might therefore reduce the risk of T-helper-2-mediated allergic responses in fetuses and neonates (Kalliomäki et al., 2001). Microbes and their antigens interact directly after birth and therefore stimulate the early development of gut-associated lymphoid tissue (Salminen et al., 1998a). Atopic disease includes initially atopic dermatitis which is followed by the development of asthma and allergic rhinitis. Atopic disease is believed to be the cause of all allergic diseases (Spergel & Paller, 2003). An abnormal immune response leads to an IgE response to common environmental allergens and therefore Th2-type cytokines are over produced (Miraglia del Giudice et al., 2003). Several clinical trials showed that probiotics can be used in the treatment of clinical conditions related to atopic disease (Table 21).

In conclusion these studies showed that probiotic administration reduced atopic inflammation and enhanced anti-inflammatory markers. However, these are only the studies with positive results. Possible explanations for different results could be the use of different probiotic strains and dosages. Microbial responsiveness and the tendency of allergic reactions are influenced by host factors and environmental factors influence colonization and the development of immunity (Prescott & Björkstén, 2007). Probiotic consumption during late pregnancy also reduced allergies in infants (Kalliomäki et al., 2001, Kukkonen et al., 2007). Insufficient data exists for probiotic treatment of allergic diseases (Prescott & Björkstén, 2007). Although positive results were observed for atopic disease, more clinical studies are needed to confirm this.

### *8.7 Reduction of cholesterol*

Cardiovascular disease is a major problem in Western countries and elevated total serum cholesterol (hypercholesteremia), mainly the low density lipoprotein (LDL)-cholesterol, and elevated fasting triglycerides are high risk factors (Hokanson & Austin, 1996). Drug therapy for the management of serum cholesterol and triglycerides levels is expensive and a low fat/low-saturated fat diet is used as an alternative (Taylor & Williams, 1998). Diets are, however, difficult to maintain and new dietary therapies including soluble fibers, soy protein, plant sterols, probiotics and prebiotics are of great interest (Taylor & Williams, 1998). The serum cholesterol levels of Maasai warriors in Africa are low and it was noticed that large amounts of *Lactobacillus* fermented milk are regularly consumed (Mann, 1974, Sharper et al., 1963). Since then, many researchers investigated the effect of fermented milk products containing *Lactobacillus* spp. and/or *Bifidobacterium* spp. on hypercholesteremia (Tables 22-25).

Table 21

## Clinical trials of probiotic treatment of atopic dermatitis

Probiotics	Subjects	Treatment	Effect	Reference(s)
<i>L. rhamnosus</i> GG (LGG)	Infants with cow milk allergy	Administration of hydrolyzed whey formula during first month	↓ atopic eczema ↓ fecal concentration of $\alpha_1$ -antitrypsin and tumor necrosis factor- $\alpha$ (anti-inflammatory properties)	Majamaa & Isolauri (1997)
LGG, <i>B. lactis</i> Bb-12	27 infants, atopic eczema during breast-feeding	Administration of hydrolyzed whey formula during first month	↓ SCORAD <sup>a</sup> -score ↓ eosinophil protein x in urine	Isolauri et al. (2000)
LGG	9 children with AD and cow milk allergy	Supplementation to diet for 4 wks	↑ IL-10	Pessi et al. (2000)
LGG	Mother with family history of atopic eczema, their infants after birth for 6 mo.	2 capsules per day for 2-4 wks, human milk	↓ atopic eczema vs. placebo	Kalliomäki et al. (2001)
LGG	Infants with atopic dermatitis	Administration for 4 wks	↑ Th1-IFN- $\gamma$ responses	Pohjavuori et al. (2004)
<i>L. paracasei</i> -33	80 children with perennial allergic rhinitis	Administration of fermented milk for 30 days	↑ quality of life	Wang et al. (2004)
<i>L. fermentum</i> PCC	Infants with atopic dermatitis	Administration for 4 wks	↑ Th1-IFN- $\gamma$ responses	Prescott et al. (2005)
LGG	6 months old IgE sensitized infants with atopic dermatitis	Administration for 4 wks	↓ SCORAD <sup>a</sup> -score	Viljanen et al. (2005)
LGG and LC705	Infants with 1 or both parents with allergic disease	Administration twice daily, 6 mo after birth	↓ atopic eczema	Kukkonen et al. (2007)

<sup>a</sup>SCORAD index = Scoring Atopic Dermatitis, ↑ = increase, ↓ = decrease

Reports from the *in vivo* trials show that total serum cholesterol, triglyceride levels, LDL-cholesterol, LDL/HDL ratio and blood pressure were reduced and HDL-cholesterol increased. Possible mechanisms include the production of short-chain fatty acids in the gut after fermentation of indigestible carbohydrates by probiotic bacteria. The synthesis of hepatic cholesterol and redistribution of cholesterol from plasma to the liver are inhibited leading to a

decrease in systematic levels of blood levels (Pereira & Gibson, 2002). Probiotic bacteria can also interfere with cholesterol absorption. Cholesterol is not taken up by the bacteria and deconjugated bile acids co-precipitate with cholesterol at a pH of less than 5.5. This affects the metabolism of cholesterol as the liver will convert cholesterol into new bile acids to compensate for the loss of bile acids (Brashears et al., 1998). The mechanisms are strain dependent and the exact mechanisms for cholesterol reduction is still unclear (De Roos & Katan, 2000).

Table 22

*In vitro* tests to study the effect of probiotic bacteria on cholesterol levels

Probiotic	<i>In vitro</i> test	Effect	Reference(s)
<i>L. acidophilus</i> RP32	Removal of cholesterol from culture medium	Cholesterol was removed by deconjugation of bile salts	Klaver & van der Meer (1993)
<i>L. casei</i> N19, <i>L. casei</i> E5, <i>L. acidophilus</i> L1 <i>L. acidophilus</i> ATCC 43121	Deconjugating bile salts; removing cholesterol from MRS at pH 6 or without pH control	60 to 90 % bile salts were deconjugated by all 4 strains. Little cholesterol was removed by the 2 <i>L. acidophilus</i> strains. <i>L. casei</i> strains removed up to 60 µg of cholesterol/ml at no pH control.	Brashears et al. (1998)
<i>L. acidophilus</i> strains	Removal of cholesterol from culture medium supplemented with bile acids	57 % removal of cholesterol	Lin & Chen (2000)
<i>L. plantarum</i> PH04	Bile salt hydrolase activity	Activity in stationary phase	Nguyen et al. (2007)

Table 23

*In vivo* trails with rats to study the effect of probiotic bacteria on cholesterol

Probiotic	Treatment	Effect	Reference(s)
<i>L. reuteri</i> CRL 1098	Probiotic supplementation to feeding of Swiss albino rats	↓ 40 % triglycerides ↑ 20 % in ratio of high density lipoprotein (HDL) to LDL	Taranto et al. (1998)
<i>Lactobacillus casei</i> TMC0409, <i>Streptococcus thermophilus</i> TMC 1543	Fermented milk supplemented with whey protein concentrate; daily intake	↓ total serum cholesterol	Kawase et al. (2000)
<i>L. plantarum</i> PH04	12 male mice; probiotic supplementation for 2wks	↓ 7 % serum total cholesterol ↓ 10 % triglycerides	Nguyen et al. (2007)

↑ = increase, ↓ = decrease

Table 24

*In vivo* trials with pigs to study the effect of probiotic bacteria on cholesterol

Probiotic	Treatment	Effect	Reference(s)
<i>L. acidophilus</i> ATCC 43121	33 Yorkshire barrows (92 kg). Feeding supplemented with probiotic plus 0.7 % /1.4 % calcium) for 15 days	↓ 11.8 % total cholesterol and ↓ 23.9 % in serum bile acids	De Rodas et al. (1996)
<i>L. johnsonii</i> BFE 1059 and BFE 1061 and <i>L. reuteri</i> BFE 1058	6 male Göttingen minipigs between 3 and 6 years (55.3 kg). Probiotic supplementation for 5 wks	↓ serum cholesterol	Du Toit et al. (1998)

↑ = increase, ↓ = decrease



Table 25

Clinical trials with human adults to study the effect of probiotic bacteria on cholesterol

Probiotic	Subjects	Treatment	Effect	Reference(s)
<i>E. faecium</i> , <i>S. thermophilus</i> (Gaio®)	58 males	200 ml fermented milk for 6 wks	↓ 6 % triglyceride ↓ 10 % LDL	Agerback et al. (1995)
<i>L. acidophilus</i> (Actimel Cholesterol Control® yoghurt)	30 males	3 x 125 ml fermented milk for 2 x 3 wks	↓ 4.4 % triglyceride ↓ 5.4 % LDL ↓ 5.3 % LDL/HDL ratio	Schaafsma et al. (1998)
<i>E. faecium</i> , <i>S. thermophilus</i> (Gaio®)	11 males, 21 females	200 g fermented milk for 8 wks	↓ 5.3 % triglyceride ↓ 6.15 % LDL	Bertolami et al. (1999)
<i>Lactobacillus casei</i> TMC0409, <i>Streptococcus thermophilus</i> TMC 1543	20 males	Fermented milk twice daily for 8 wks	↑ HDL ↓ triglyceride level ↓ systolic blood pressure	Kawase et al. (2000)
<i>E. faecium</i> , <i>S. thermophilus</i> (Gaio®)	20 males, 50 females	450 ml fermented milk for 8 wks	↓ 8.4 % LDL	Larsen et al. (2000)
<i>L. acidophilus</i> , <i>B. longum</i>	29 females	300g yoghurt for 51 days	↓ 50 % LDL	Schaarmann et al. (2001)

↑ = increase, ↓ = decrease

### 8.8 Alleviation of lactose intolerance

The enzyme, beta-galactosidase (lactase), hydrolyses lactose to glucose and galactose (Savaiano & Levitt, 1987). The enzyme is produced in the gastro-intestinal tract for the first years of life. In specific ethnic groups, including blacks, Asians and South Americans, the levels of this enzyme decrease with age, resulting in lactose intolerance (inability to hydrolyze lactose) (Andersson et al., 2001). Lactose intolerance can also develop secondary to an infection of the small intestine or destruction of mucosal cells due to other causes or other infections or conditions, such as diarrhoea, AIDS or giardiasis, especially in children. Small bowel surgery or prolonged disuse of the gastro-intestinal tract may also affect lactase activity (Tyrus, 1996). The symptoms of lactose intolerance include abdominal pain, flatulence, or diarrhoea because the lactose behaves like an osmotic and non-digestible carbohydrate (Pfeifer & Rosat, 1999, Roberfroid, 2000). Probiotic bacteria used to produce fermented milk or yoghurt products produce bacterial  $\beta$ -galactosidase in the intestine and stomach where lactose is degraded (Kopp-

Hoolihan, 2001). Although lower concentrates are produced in the gastro-intestinal tract than in the yoghurt starter cultures of *L. bulgaricus* and *S. thermophilus*, the resulting  $\beta$ -galactosidase levels are present in the gastro-intestinal tract for longer time periods, facilitating lactose hydrolysis (Sanders, 1993). Lactose from yoghurt and milk containing the probiotic *L. acidophilus* was better absorbed by subjects with low  $\beta$ -galactosidase activity. The symptoms of lactose intolerance were fewer and bacterial fermentation of undigested lactose was also evident in breath hydrogen concentrations (Table 26). Dairy products or other products containing the mentioned probiotic bacteria can be included in the diet of people suffering from lactose intolerance to prevent/reduce the symptoms.

## 9. Conclusion and future perspectives

Different techniques were described and proposed to evaluate lactic acid bacteria for probiotic properties. Several bacterial strains were well studied in the different assays and clinical trials and can be defined as reliable probiotics. From the scientific data it is also evident that probiotic properties are strain specific and that these strains do not meet all the probiotic criteria. *In vitro* assays can give an indication of probiotic properties and can be the first part of the selection process. This process might be refined in future by using genomic analysis. However, clinical trials are the most reliable method to ensure probiotic validity. This will reduce uncertainties about the use of probiotics as health supplements and functional foods. Until these regulations are strictly followed, probiotics will battle to be fully recognized in promoting human health.

## References

- Aatourri, N., Bouras, M., Tome, D., Marcos, A., Lemonnier, D., 2002. Oral ingestion of lactic acid bacteria by rats increases lymphocyte proliferation and interferon- $\gamma$  production. *British Journal of Nutrition* 87, 367–373.
- Adlerberth, I., Ahn , S., Johansson, M.-L., Molin, G., Hanson, L.A., Wold, A.E., 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Applied and Environmental Microbiology* 62, 2244-2251.

- Agerbaek, M., Gerdes, L.U., Richelsen, B., 1995. Hypocholesterolemic effect of a new fermented milk product in healthy middle-aged men. *European Journal of Clinical Nutrition* 49, 346-352.
- Alakomi, H.-L., Skyttä, E., Saareela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M., 2000. Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology* 66, 2001-2005.
- Alam, M., Miyoshi, S., Yamamoto, S., Tomochika, K., Shinoda, S., 1996. Expression of virulence-related properties by and intestinal adhesiveness of *Vibrio mimicus* strains isolated from aquatic environments. *Applied and Environmental Microbiology* 62, 3871-3874.
- Alander, M., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., von Wright, A., 1997. Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. *Letters in Applied Microbiology* 24, 361-364.
- Allen, W.D., Linggood, M.A., Porter, P., 1996. *Enterococcus* organisms and their use as probiotics in alleviating irritable bowel syndrome symptoms. European Patent 0508701 (B1).
- Altermann, E., Russell, W.M., Azcarate-Peril, M.A., Barrangou, R., Buck, B.L., McAuliffe, O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R., Klaenhammer, T.R., 2005. Complete genome sequence of the probiotic *Lactobacillus acidophilus* NCFM, 102. *Proceedings of the National Academy of Sciences of the United States of America* 11, 3906-3912.
- Alvarez-Olmos, M., Oberhelman, R.A., 2001. Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. *Clinical Infectious Diseases* 32, 1567-1576.
- Andersson, H., Asp, N.-G., Bruce, A., Roos, A., Wadström, T., Wold, A.E., 2001. Health effects of probiotics and prebiotics. A literature review on human studies. *Scandinavian Journal of Nutrition* 45, 58-75.
- Andreu, A., Stapleton, A.E., Fennell, C.L., Hillier, S.L., W. E. Stamm, W.E., 1995. Hemagglutination, adherence and surface properties of vaginal *Lactobacillus* species. *Journal of Infectious Diseases* 171:1237-1243.
- Arthur, M., Courvalin, P., 1993. Genetics and mechanism of glycopeptide resistance enterococci. *Antimicrobial Agents in Chemotherapy* 37, 1563-1571.

- Arvola, I., Latho, K., Torkkeli, S., 1999. Prophylactic *Lactobacillus* GG reduces antibiotic-associated diarrhoea in children with respiratory infections: a randomized study. *Pediatrics* 104, 64.
- Aso, Y., Akazan, H., 1992. Prophylactic effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer. *Urology International* 49, 125–129.
- Aso, Y., Akaza, H., Kotake, T., Tsukamoto, T., Imai, K., 1995. Preventive effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer in a double blind trial. *European Urology* 27, 104–109.
- Åvall-Jääskeläinen, S., Kylä-Nikkilä, K., Kahala, M., Mäikkulainen-Lahti, T., Palva, A., 2002. Surface display of foreign epitopes on the *Lactobacillus brevis* S-layer. *Applied and Environmental Microbiology* 68, 5943–5951.
- Åvall-Jääskeläinen, S., Palva, A., 2005. *Lactobacillus* surface layers and their applications. *FEMS Microbiology Reviews* 29, 511-529.
- Axelsson, L.T., Chung, T.C., Dobrogosz, W., Lidgren, S.E., 1989. Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. *Microbial Ecology in Health and Disease* 2, 131-136.
- Aymerich, T., Holo, H., Håvarstein, L.S., Hugas, M., Garriga, M., Nes, I.F., 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Applied and Environmental Microbiology* 62, 1676-1682.
- Azcarate-Peril, M., Altermann, E., Hoover-Fitzula, R.L., Cano, R., Klaenhammer, T.R., 2004. Identification and inactivation of genetic loci involved with *Lactobacillus acidophilus* acid tolerance. *Applied and Environmental Microbiology* 70, 5315-5322.
- Baccigalupi, L., Donato, A.D., Parlato, M., Luongo, D., Carbone, V., Rossi, M., Ricca, M. and De Felice, M., 2005. Small surface-associated factors mediate adhesion of a food-isolate strain of *Lactobacillus fermentum* to Caco-2 cells. *Research in Microbiology* 156, 830-836.
- Balla, E., Dicks, L.M.T., Du Toit, M., van der Merwe, M.J., Holzapfel, W.H., 2000. Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. *Applied and Environmental Microbiology* 66, 1298-1304.

- Bampton, P.A., Dinning, P.G., Kennedy, M.L., Lubowski, D.Z., Cook, I.J., 2002. The proximal colonic motor response to rectal mechanical and chemical stimulation. *American Journal of Physiology* 282, G443–G449.
- Barker, D.C., Thorne, K.J.I., 1970. Spheroplasts of *Lactobacillus casei* and the cellular distribution of bactoprenol. *Journal of Cell Science* 7, 755–785.
- Bazzocchi, G., Gionchetti, P., Almerigi, P.F., Amadini, C. and Campirei, M., 2002. Intestinal microflora and oral bacteriotherapy in irritable bowel syndrome. *Digestive and Liver Disease* 34, S48–S53.
- Begley, M., Gahan, C.G., Hill, C., 2000. Bile stress response in *Listeria monocytogenes* LO28: Adaptation, cross-protection, and identification of genetic loci involved in bile resistance. *Applied and Environmental Microbiology* 68, 6005-6012.
- Bellomo, G., Mangiagle, A., Nicastro, L., Frigerio, G., 1980. A controlled double-blind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in pediatrics. *Current Therapeutic Research* 28, 927–934.
- Bengmark, S., 2003. Use of some pre-, pro-, and synbiotics in critically ill patients. *Best Practice and Research: Clinical Gastroenterology* 17, 833-848.
- Benn, M., Hagelskjaer, L.H, Tvede, M., 1997. Infective endocarditis, 1984 through 1993: a clinical and microbiological survey. *Journal of International Medicine* 242, 15-22.
- Bernet, M.F., Brassart, D., Neeser, J.R., Servin, A.L., 1994. *Lactobacillus acidophilus* La1 binds to cultured human intestinal cell lines and inhibits cell-attachment and cell invasion by enterovirulent bacteria. *Gut* 35, 483-489.
- Bernet-Camard, M.F., Lievin, V., Brassart, D., Neeser, J.R., Servin, A.L., Hudault, S., 1997. The human *Lactobacillus acidophilus* strain La1 secretes a nonbacteriocin antibacterial substance(s) active *in vitro* and *in vivo*. *Applied and Environmental Microbiology*, 63, 2747-2753.
- Bertazzoni-Minelli, E., Benini, A., Marzotto, M., Sbarbati, A., Ruzzenente, O., Ferrario, R., Hendriks, H., Dellaglio, F., 2004. Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional foods. *International Dairy Journal* 14, 723-736.
- Bertolami, M.C., Faludi, A.A., Batlouni, M., 1999. Evaluation of the effects of a new fermented milk product (Gaio) on primary hypercholesterolemia. *European Journal of Clinical Nutrition* 53, 97-101.

- Bianchi, M.A., Del Rio, D., Pellegrini, N., Sansebastiano, G., Neviani, E., Brighenti, F., 2004. A Fluorescent-based method for the detection of adhesive properties of lactic acid bacteria to Caco-2 cells. *Letters of Applied Microbiology* 39, 301-305.
- Bjorksten, B., Naaber, P. Sepp, E., Mikelsaar, M., 1999. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clinical and Experimental Allergy* 29, 342–346.
- Blom, H., Mörtvedt, C., 1991. Anti-microbial substances produced by food associated microorganisms. *Biochemical Society Transactions* 19, 694-698.
- Blum, S., Reneiro, R., Schiffrin, E.J., Crittenden, R., Matitila-Sandhom, T., Ouwehand, A.C., Salminen, S., von Wright, A., Saarela, M., Saxelin, M., Collins, K., Morelli, L., 1999. Adhesion studies for probiotics: need for validation and refinement. *Food Science Technology* 10, 405-410.
- Bomba, A., Nemcova, R., Kastel, R., 1996. Interaction of *Lactobacillus* spp. and enteropathogenic *Escherichia coli* under *in vitro* and *in vivo* conditions. *Veterinary Medicine (Praha)*, 41, 155-158.
- Boot, H.J., Kolen, C.P.A.M., Van Noort, J.M., Pouwels, P.H., 1993. S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli* and nucleotide sequence of the corresponding gene. *Journal of Bacteriology* 175, 6089–6096.
- Boot, H.J., Kolen, C.P.A.M., Pouwels, P.H., 1995. Identification, cloning, and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein of this species. *Journal of Bacteriology* 177, 7222–7230.
- Boot, H.J., Pouwels, P.H., 1996. Expression, secretion and antigenic variation of bacterial S-layer proteins. *Molecular Microbiology* 21, 1117–1123.
- Boot, H.J., Kolen, C.P.A.M., Pot, B., Kersters, K., Pouwels, P.H., 1996. The presence of two S-layer protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*. *Microbiology* 142, 2375–2384.
- Booth, M.C., Bogie, C.P., Sahl, H.-G., Siezen, R.J., Hatter, K.L., Gilmore, M.S., 1996. Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. *Molecular Microbiology* 21, 1175-1184.
- Borruel, N., Carol, M., Casellas, F., Antolín, M., de Lara, F., Espin, E., Naval, J., Guarner, F., Malagelada, J.R., 2002. Increased mucosal tumor necrosis factor a production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. *Gut* 51, 659–664.

- Borruel, N., Casellas, F., Antolin, M., Llopis, M., Carol, M., Espiin, E., 2003. Effects of nonpathogenic bacteria on cytokine secretion by human intestinal mucosa. *American Journal of Gastroenterology* 98, 865–870.
- Brashears, M.M., Jaroni, D., Trimble, J., 1993. Isolation, secretion, and characterization of lactic acid bacteria for a competitive exclusion product to reduce shedding of *Escherichia coli* O157: H7 in cattle. *Journal of Food Protection* 66, 355-363.
- Brashears, M.M., Gilliland, S.E., Buck, L.M., 1998. Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. *Journal of Dairy Science* 81, 103–2110.
- Braun, P.C., 1999. Nutrient uptake by *Candida albicans*: the influence of cell surface mannoproteins. *Canadian Journal of Microbiology* 45, 353-359.
- Breton, Y.L., Maze, A., Hartke, A., Lemarinier, S., Auffray, Y. and Rince, A., 2002. Isolation and characterization of bile salts-sensitive mutants of *Enterococcus faecalis*. *Current Microbiology* 45, 434-439.
- Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O.P., Sahl, H., de Kruijff, B., 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* 286, 2361-2364.
- Bron, P.A., Grangette, C., Meroenier, A., de Vos, W.M., Kleerebezem, M., 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastro-intestinal tract of mice. *Journal of Bacteriology* 186, 5721-5729.
- Bron, P.A., Hoffer, S.M., Van Swam, I.I., de Vos, W.M., Kleerebezem, M., 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. *Applied and Environmental Microbiology* 70, 310-317.
- Bron, P.A., Meijer, M., Bongers, R., de Vos, W.M., Kleerebezem, M., 2004. Competitive population dynamics of gene mutants of *Lactobacillus plantarum* in the gastro-intestinal tract of mice. In: Bron, P.A. (Ed.), *The molecular response of Lactobacillus plantarum to intestinal passage and conditions*, Wageningen, Wageningen Centre for Food Sciences.
- Bron, P.A., Molenaar, D., de Vos, W.M., Kleerebezem, M., 2006. DNA micro-array based identification of bile-responsive genes in *Lactobacillus plantarum*. *Journal of Applied Microbiology* 100, 728-738.
- Bouhnik, Y., Flourie, B., Andrieux, C., Bisetti, N., Rambaud, J.C., 1996. Effects of *Bifidobacterium* sp. fermented milk ingested with or without inulin on colonic



- Bifidobacteria and enzymatic activities in healthy humans. *European Journal of Clinical Nutrition* 50, 269–273.
- Budde, B.B., Rasch, M.A., 2001. A comparative study on the use of flow cytometry and colony forming units for assessment of the antibacterial effect of bacteriocins. *International Journal of Food Microbiology* 63, 65-72.
- Callegari, M.L., Riboli, B., Sanders, J.W., Cocconcelli, P.S., Kok, J., Venema, G., Morelli, L., 1998. The S-layer gene of *Lactobacillus helveticus* CNRZ 892: cloning, sequence and heterologous expression. *Microbiology* 144, 719–726.
- Candela, M., Seibold, G., Vitali, B., Lachenmaier, S., Eikmanns, B., Brigidi, P., 2005. Real-time PCR quantification of bacterial adhesion to Caco-2 cells: Competition between bifidobacteria and enteropathogens. *Research in Microbiology* 156, 887-895.
- Canducci, F., Armuzzi, A., Cremonini, F., Cammarota, G., Bartolozzi, F., Pola, P., Gasbarrini, G., Gasbarrini, A., 2000. A lyophilized and inactivated culture of *Lactobacillus acidophilus* increases *Helicobacter pylori* eradication rates. *Alimentary Pharmacology and Therapeutics* 1625-1629.
- Cannon, J. P., Lee, T.A., Bolanos, J.T., Danziger, L.H., 2005. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. *European Journal of Clinical Microbiology* 24, 31-40
- Carlson, E., 1998. Irritable bowel syndrome. *Nurse Practitioner* 23, 82–91.
- Carlsson, J., Iwami, Y., Yamada, T., 1983. Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase thiocyanate-hydrogen peroxide. *Infection and Immunity* 40, 70-80.
- Casaus, P., Nilsen, T., Cintas, L.M., Nes, I.F., Hernández, P.E., Holo, H., 1997. Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. *Microbiology* 143, 2287-2294.
- Chadwick, V.S., Gaginella, T.S., Carlson, G.L., Debonnie, J.C., Phillips, S.F., Holmann, A.F., 1979. Effect of molecular structure on bile acid-induced alterations in absorptive function, permeability, and morphology in the perfused rabbit colon. *Journal of Laboratory and Clinical Medicine* 94, 661–674.
- Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K., 1998. Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastro-intestinal tract. *Journal of Applied Microbiology* 84, 759-768.



- Chauvier, G., Coconier, M.H., Kerneis, S., 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. *Journal of General Microbiology* 138, 1689-1696.
- Chauvière, G., Coconnier, M.H., Kerneis, S., 1992. Competitive exclusion of diarrhoeagenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat-killed *Lactobacillus*. *FEMS Microbiology Letters* 91, 3, 213-217.
- Chen, G., Russel, J.B., 1989. Transport of glutamine by *Streptococcus bovis* and conversion of glutamine to pyroglutamic acid and ammonia. *Journal of Bacteriology* 171, 2981-2985.
- Chen, A., Xu, J., Shuai, J., Chen, J., Zhang, Z., Fang, W., 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* 0157:H7 and *Salmonella typhimurium*. *International Journal of Food Microbiology* 115, 307-312.
- Cherrington, C.A., Hinton, M., Mead, G.C., Chopra, I., 1991. Organic acids: chemistry, antibacterial activity and practical application. *Advanced Microbiology and Physics* 32, 87-108.
- Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., Zervos, M.J., 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. *Antimicrobial Agents in Chemotherapy* 37, 2474-2477.
- Chung, T.C., Axelsson, L.T., Lindgren, S.E., Dobrogosz, W.J., 1989. *In vitro* studies on reuterin synthesis by *Lactobacillus reuteri*. *Microbial Ecology in Health and Disease* 2, 137-144.
- Cintas, L.M., Rodriguez, J.M., Fernandes, M.F., Sletten, K., Nes, I.F., Hernandez, P.E., Holo, H., 1995. Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. *Applied and Environmental Microbiology* 61, 2643-2648.
- Clancy, R., 2003. Immunobiotics and the probiotic evolution. *FEMS Immunology Medical Microbiology* 38, 9-12.
- Clewell, D.B., 1993. Bacterial sex pheromone-induced plasmid transfer. *Cell* 73, 9-12.
- Clewell, D.B., An, F.Y., Flannagan, S.E., Antiporta, M., Dunny, G.M., 2000. Enterococcal sex pheromone precursors are part of signal sequences for surface lipoproteins. *Molecular Microbiology* 35, 246-247.
- Coconnier, M.H., Lieven, V., Lorrot, M., Servin, A.L., 2000. Antagonistic activity of *Lactobacillus acidophilus* LB against intracellular *Salmonella enterica* serovar

- Typhimurium infecting human enterocyte-like Caco-2/TC-7 cells. *Applied and Environmental Microbiology* 66, 1152-1157.
- Cohen, P.S., Laux, D.C., 1995. Bacterial adhesion to and penetration of intestinal mucus *in vitro*. *Methods in Enzymology* 253, 309-314.
- Commane, D. Hughes, R. Shortt, C., Rowland, I., 2005. The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mutation Res.* 591, 276-289.
- Condon, S., 1987. Responses of lactic acid bacteria to oxygen., 1987. *FEMS Microbiology Reviews* 46, 269-280.
- Conway, P.L., Gorbach, S.L., Goldin, B.R., 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cell. *Journal of Dairy Science* 70, 1-12.
- Coque, T.M., Patterson, J.E., Steckelberg, J.M., Murray, B.E., 1995. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from the feces of hospitalized and community-based persons. *Journal of Infectious Diseases* 171, 1223-1229.
- Cotter, P.D., Hill, C., 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews* 67, 429-53.
- Courvalin P, Trieu-Cuot P., 2001. Minimizing potential resistance: The molecular view. *Clinical Infectious Diseases* 33:S138-46.
- Courvalin, P., 2006. Antibiotic resistance: The pros and cons of probiotics. *Digestive and Liver Disease* 38, (suppl.2), S261-265.
- Cross, M.L., 2002. Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunology and Medical Microbiology* 34, 245-253.
- Cummings, J.H., 1997. Anatomy, bacteriology and function. In: *The large intestine in nutrition and disease*. (Ed.), Institute Danone.
- Dambekodi, P.C., Gilliland, S.E., 1998. *Journal of Dairy Science* 81, 4344-4349.
- Dani, C., Biadaioli, R., Bertini, G., Martelli, E., Rubaltelli, F.F., 2002. Probiotics feeding in prevention of urinary tract infection, bacterial sepsis, and necrotizing enterocolitis in preterm infants. A prospective double-blind study. *Biology of the Neonate* 82, 103-108.
- Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology* 82, 1 –11.

- Davies, J., 1997. Origins, acquisition and dissemination of antibiotic resistance determinants. In: D.J. Chadwick, J. Goode, J. (Eds.), Antibiotic Resistance: Origins, Evolution, Selection and Spread, Ciba Foundation Symposium, vol. 207. Wiley, Chichester, 15 -27.
- Del Piano, M., Morelli, L., Strozzi, G.P., Allestina, S., Barab, M., Deidda, F., Lorenzini, P., Ballaré, M., Montino, F., Orsello, M., Sartori, M., Garelo, E. Carmagnola, S., Pagliarulo, M., Capurso, L., 2006. Probiotics: from researcher to consumer. Digestive and Liver Disease 38 (Suppl.2), S248-S255.
- De Rodas, B.Z., Gilliland, S.E., Maxwell, C.V., 1996. Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. Journal of Dairy Science 79, 2121–2128.
- De Roos, N.M., Katan, M.B., 2000. Effect of probiotic bacteria on diarrhoea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. American Journal of Clinical Nutrition 71, 405-411.
- De Smet, I., Van Hoorde, L., De Saeyer, N., Van de Woestyme, M., Vestraete, W., 1994. *In vitro* study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of lowering through enhanced BSH activity. Microbial Ecology in Health and Disease 7, 315–329.
- Devlieghere, F., Debevere, J., 2000. Influence of dissolved carbon dioxide on the growth of spoilage bacteria. Lebensmittel- und Wissenschaft-Technologie. 33, 531-537.
- De Vos, W.M., Kuipers, O.P., van der Meer, J.R., Siezen, R.J., 1995. Maturation pathway of nisin and other lantibiotics: post-translationally modified antimicrobial peptides exported by Gram-positive bacteria. Molecular Microbiology 17, 427-437.
- De Vos, W.M.; Bron, P.A., Kleerebezem, M., 2004. Post-genomics of lactic acid bacteria and other food-grade bacteria to discover gut functionality. Current opinion in Biotechnology 15, 86-93.
- De Vriese, M.C., Vaughan, E.E., Kleerebezem, M., de Vos, W.M., 2006. *Lactobacillus plantarum*-survival, functional and potential probiotic properties in the human intestinal tract. International Dairy Journal 16, 1018-1028.
- De Vuyst, L., Vandamme, E.J., 1994. Bacteriocins of lactic acid bacteria. London, Chapman & Hall, 91-142.
- Dobrogosz, W.J., Casas, I.A., Pagano, G.A., Talarico, T.L., Sjöberg, B.-M., Karlsson, M., 1989. *Lactobacillus reuteri* and the enteric microbiota. In: The Regulatory and Protective Role of the Normal Microflora. Norin, E., (Ed.), Stockton Press, New York, pp. 283-292.

- Donohue, D.C., Salminen, S., 1996. Safety assessment of probiotic bacteria. *Asia Pacific Journal of Clinical Nutrition* 5, 25–28.
- Donohue, D., Salminen, S., Marteau, P., 1998. Safety of probiotic bacteria. In: Salminen, S., von Wright, A. (Eds.), *Lactic Acid Bacteria*. Marcel Dekker, New York, pp. 369–384.
- Donnet-Hughes, A., Rochat, F., Serrant, P., Aeschlimann, J.M., Schiffrin, E.J., 1999. Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. *Journal of Dairy Science* 82, 863-869.
- D’Souza, A.L., Rajkumar, C., Cooke, J., Bullpitt, C.J., 2002. Probiotics in the prevention of antibiotic-associated diarrhoea: meta analysis. *British Medical Journal* 324, 1361-1364.
- Dugas, B., Mercenier, A., Lenoir-Wijnkoop, I., Arnaud, C., Dugas, N. and Postaire, E., 1999. Immunity and probiotics. *Trends in Immunology Today* 20,387-390.
- DuPont, H.L., Ericsson, C.D., 1993. Prevention and treatment of traveler’s diarrhoea. *The New England Journal of Medicine* 328, 1821-1827.
- Dutka-Malen, S., Evers, S., Courvain, P., 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *Journal of Clinical Microbiology* 33, 24–27.
- Du Toit, M., Franz, C.M.A.P., Dicks, L.M.T., Schillinger, U., Haberer, P., Warlies, B., Ahrens, F., Holzapfel, W.H., 1998. Characterization and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol level, faeces pH and faeces moisture content. *International Journal of Food Microbiology* 40, 93–104.
- Eaton, T.J., Gasson, M.J., 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology* 67, 1628-1635.
- Eaton, T.J., Gasson, M.J., 2002. A variant enterococcal surface protein Espfm in *Enterococcus faecium*, distribution among food, commensal, medical, and environmental isolates. *FEMS Microbiology Letters* 216, 269-275.
- Eijsink, V.G., Axelsson, L., Diep, D.B., Havarstein, L.S., Holo, H., Nes, I.F., 2002. Production of classII bacteriocins by lactic acid bacteria: an example of biological warfare and communication. *Antonie Van Leeuwenhoek* 81, 639-654.
- Elo, S., Saxelin, Salminen, S., 1991. Attachment of *Lactobacillus casei* GG to human colon cell line Caco-2: comparison with other dairy strains. *Letters in Applied Microbiology* 13, 154-156.

- Elsner, H.-A., Sobottka, I., Mack, D., Claussen, M., Laufs, R., Wirth, R., 2000. Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium* blood culture isolates. European Journal of Clinical Microbial Infectious Diseases 19, 39-42.
- European Commission, 2004. Position paper of the Scientific Committee on Animal Nutrition on Safety Assessment and Regulatory Aspects of Micro-organisms in Feed and Food Applications.[http://europa.eu.int/comm/food/fs/sc/scan/out85\\_en.pdf](http://europa.eu.int/comm/food/fs/sc/scan/out85_en.pdf).
- Fang, H., Elina, T., Heikki, A., Salminen, S., 2000. Modulation of humoral immune response through probiotic intake. FEMS Immunology Medical Microbiology 29, 47-52.
- FAO/WHO, 2002. Guidelines for the Evaluation of Probiotics in Food. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. (<ftp://ftp.fao.org/es/esn/food/wgreport2.pdf>)
- Felley, C.P., Corthesy-Theulaz, I., Rivero, J.L., Sipponen, P., Kaufmann, M., Bauerfeind, P., Wiesel, P.H., Brassart, D., Pfeifer, A., Blum, A.L., Michetti, P., 2001. Favourable effect of an acidified milk (LC-1) on *Helicobacter pylori* gastritis in man. European Journal of Gastroenterologic Hepatology 13, 25-29.
- Fernández, M.F., Boris, S., Barbés, C., 2003. Probiotic properties of human lactobacilli strains to be used in the gastro-intestinal tract. Journal of Applied Microbiology 94, 449-455.
- Fioramonti, J., Theodorou, V., Bueno, L., 2003. Probiotics: what are they? What are their effects on gut physiology? Best Practice & Research Clinical Gastroenterology 17,, 711-724.
- Floch, M.H., Binder, H.J., Filburn, B., Gershengoren, W., 1972. The effect of bile acids on intestinal microflora. American Journal of Clinical Nutrition 25, 1418-1426.
- Floch, M.H., 2002. Bile salts, intestinal microflora and enterohepatic circulation. Digestive and Liver Disease 34 (Suppl2), S54-57.
- Fogh, J.J., Fogh, J.M., Orfeo, T., 1977. One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. Journal of National Cancer Institute 59, 221-226.
- Forestier, C., De Champs, C., Vatoux, C., Joly, B., 2001. Probiotic activities of *Lactobacillus casei rhamnosus*: *in vitro* adherence to intestinal cells and antimicrobial properties. Research in Microbiology 152, 167-173.
- Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. International Journal of Food Microbiology 106, 1-24.

- Fraise, A.P., 1996. The treatment and control of vancomycin resistant enterococci. *Journal of Antimicrobial Chemotherapy* 38, 753–756.
- Francis, K.P., Joh, D., Bellinger-Kawahara, C., Hawkinson, M.J., Purchio, T.F., Contag, P.R., 2000. Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel luxABCDE construct. *Infection and Immunity* 68, 3594-3600.
- Francis, K.P., Yu, J., Bellinger-Kawahara, C., Joh, D., Purchio, T.F., Hawkinson, M.J., Xiao, G., Caparon, M.G., Lipsitch, M., Contag, P.R., 2001. Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumonia* transformed with a novel Gram-positive lux transposon. *Infection and Immunity* 69, 3350-3358.
- Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E., 1999. Enterococci at the crossroads of food safety. *International Journal of Food Microbiology* 47, 1-24.
- Franz, C.M.A.P., Muscholl-Silberhorn, A.B., Yousif, N.M.K., Vancanneyt, M., Swings, J., Holzapfel, W.H., 2001. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food, *Applied and Environmental Microbiology* 67, 4385-4389.
- Franz, C.M.A.P., Grube, A., Herrmann, A., Abriouel, H., Stärke, J., Lombardi, A.Tauscher, B., Hozhapfel, W.H., 2002. Biochemical and genetic characterization of the two-peptide bacteriocin enterocin 1071 produced by *Enterococcus faecalis* FAIR-309. *Applied and Environmental Microbiology* 68, 2550-2554.
- Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H., Holzapfel, W.H., 2003. Enterococci in foods-a conundrum for food safety. *International Journal of Food Microbiology* 88, 105-122.
- Franz, C.M.A.P., Holzapfel, W.H., 2004. The genus *Enterococcus*: Biotechnological and safety issues. In: Salminen, S., von Wright, A., Ouwehand, A. (Eds.), *Lactic acid bacteria Microbiological and Functional aspects*, 3<sup>rd</sup> ed., Revised and expanded, Marcel Dekker inc.
- Frece, J., Kos, B., Svetec, I.K., Zgaga, Z., Mrša, V., Šušková, J., 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology* 982, 285-292.
- Fuller, M.E., Streger, S.H., Rothmel, R.K., Mailloux, B.J., Hall, J.A., Onstott, T.C., Fredrickson, J.K., Balkwill, D.L., DeFlaun, J.A., 2000. Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. *Applied and Environmental Microbiology* 66, 4486-4496.

- Gálvez, A., Valdiva, E., Abrioul, H., Camafeita, E., Mendez, E., Martínez-Bueno, M., Maqueda, M., 1998. Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Archives in Microbiology 171, 59-65.
- Gálvez, A., Maqueda, M., Valdiva, E., Quesada, A., Montoya, E., 1986. Characterization and partial purification of a broad spectrum antibiotic AS-48 produced by *Streptococcus faecalis*. Canadian Journal of Microbiology 32, 765-771.
- Gänzle, M.G., Höltzel, A., Walter, J., Jung, G., Hammes, W.P., 2000. Characterization of reutericyclin produced by *Lactobacillus reuteri* LTH2584. Applied and Environmental Microbiology 66, 4325-4333.
- Gänzle, M.G., Vogel, R.F., 2003. Studies on the mode of action of reutericyclin, 2003. Applied and Environmental Microbiology 69, 1305-1307.
- Garcia Lafuente, A.; Antolin, M.; Guarner, F.; Crespo, E. and Malagelada, J.R., 2001. Modulation of colonic barrier function by the composition of the commensal flora in the rat. Gut 48, 503–507.
- Gardiner, G.E., Ross, R.P., Wallace, J.M., Scanlan, F.P., Jägers, P.P.J.M., Fitzgerald, G.F., Collins, J.K., Stanton, C., 1999. Influence of a probiotic adjunct culture of *Enterococcus faecium* on the quality of Cheddar cheese. Journal of Agriculture and Food Chemistry 47, 4907-4916.
- Garrote, G.L., Delfederico, L., Bibiloni, R., Abraham, A.G., Pérez, P.F., Semorile, L., De Antoni, G.L., 2004. Lactobacilli isolated from kefir: evidence of the presence of S-layer proteins. Journal of Dairy Research 71, 222–230.
- Gaskins, H.R., 1997. Immunological aspects of host/microbiota interactions at the intestinal epithelium. In: Mackie, R.I., White, B.A. and RE Isaacson, R.E. (Eds.), Gastro-intestinal microbiology, International Thomson Publishing, New York, pp. 537–587.
- Gasser, F., 1994. Safety of lactic acid bacteria and their occurrence in human clinical infections. Bulletin De L’Institute Pasteur 92, 45–67.
- Gibson, G.R., Macfarlane, G.T., 1994. Intestinal bacteria and disease. In: Gibson, S.A.W. (Eds.), Human health-The contribution of microorganisms, Springer-Verlag, London, pp. 53-62.
- Gill, H.S., 2003. Probiotics to enhance anti-infective defences in the gastro-intestinal tract. Best Practice in Research in Clinical Gastroenterology 17, 755-773.
- Gilliland, S.E., Speck, M.L., 1977. Deconjugation of bile acids by intestinal lactobacilli. Applied and Environmental Microbiology 33, 15-18.



- Gilliland, S.E., Stanley, T.E., Bush, L.J., 1984. Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of Dairy Science* 67, 3045–3051.
- Gilliland, S.E., Walker, D.K., 1990. Factors to consider when selecting a dietary adjunct to produce a hypocholesterolemic effect in humans. *Journal of Dairy Science* 73, 905-911.
- Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R., Clewell, D.B., 1994. Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *Journal of Bacteriology* 176, 7335-7344.
- Gionchetti, P.; Rizzello, F., Campieri, M., 1999. *In vivo* effect of a highly concentrated probiotic on IL-10 pelvic pouch tissue levels. *Gastroenterology* 116, 723.
- Gionchetti, P.; Rizzello, F., Venturi, A., 2000a. Oral probiotic therapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. *Gastroenterology* 119, 305–309.
- Gionchetti, P.; Rizzello, F., Venturi, A., 2000b. Prophylaxis of pouchitis onset with probiotic therapy: a double blind, placebo controlled trial. *Gastroenterology* 118, 1214.
- Gold, O., Jordan, H.V., van Houte, J., 1975. The prevalence of enterococci in the human mouth and their pathogenicity in animal models. *Archives in Oral Biology* 20, 473-477.
- Goldin, B., Gualtieri, L., Moore, R., 1996. The effect of *Lactobacillus GG* on the initiation and promotion of DMH induced intestinal tumours in the rat. *Nutrition and Cancer* 25, 197–204.
- Gorbach, S.I., 2002. Probiotics in the Third Millennium. *Digestive and Liver Disease* 34, (Suppl.2), S2-7.
- Gopal, P.K., Prasad, J., Smart, J., Gill, H.S., 2001. *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *International Journal in Food Microbiology* 67, 207-216.
- Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., Brassart, D., 1999. Cell-surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Applied and Environmental Microbiology* 65, 1071-1077.
- Granato, D., Bergonzelli, G.E., Pridmore, R.D., Marvin, L., Rouvet, M., Corthésy-Theulaz, I.E., 2004. Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infection and Immunity* 72, 2160-2169.



- Graninger, W., Ragette, R., 1992. Nosocomial bacteremia due to *Enterococcus faecalis* without endocarditis. *Clinical Infectious Diseases* 15, 49-57.
- Greene, J.D., Klaenhammer, T.R., 1994. Factors involved in adherence of Lactobacilli to human Caco-2 cells. *Applied and Environmental Microbiology* 60, 4487-4494.
- Guandalini, S., Pensabene, L., Zikri, M.A., Dias, J.A., Casali, L.G., Hoekstra, H., Kolacek, S., Masser, K., Micetic-Turk, D., Papadopoulou, A., 2000. *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhoea: a multicenter European study. *Journal of Pediatric Gastroenterology Nutrition* 30, 54-60.
- Guslandi, M., Mezzi, G., Sorghi, M., Testoni, P.A., 2000. *Saccharomyces boulardii* in maintenance treatment of Crohn's disease. *Digestive Disease Sciences* 45, 7, 1462–1464.
- Guyton, A.C., 1991. Transport and mixing of food in the alimentary tract; Secretory functions of the alimentary tract, In: *Textbook of medical physiology*, 8<sup>th</sup> Ed. W.B. Saunders Company, Harcourt Brace Jovanovich, Inc.
- Hamilton-Miller, J.M.T., Shah, S., 1998. Vancomycin susceptibility as an aid to the identification of lactobacilli. *Letters of Applied Microbiology* 26, 153–154.
- Hancock, L.E., Gilmore, M.S., 2002. The capsular polysaccharide of *Enterococcus faecalis* and its relationship to other polysaccharides in the cell wall. *Proceedings of the National Academy of Sciences of the United States of America* 99, 1574-1579.
- Hansen, J.N., Banerjee, S., Buchman, L.W., 1989. Potential of small ribosomally synthesized bacteriocins in the design of new food preservatives, *Journal of Food Safety* 10, 119–130.
- Hansen, G., McIntire, J.J., Yeung, V.P., 2000. CD4(+) T helper cells engineered to produce latent TGF-beta 1 reverse allergen-induced airway hyperreactivity and inflammation. *Journal of Clinical Investigation* 105, 61–70.
- Hechard, Y., Sahl, H.G., 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie* 84, 545-557.
- Heyman, M., 2000. Effect of lactic acid bacteria on diarrhoeal diseases. *Journal of the American College of Nutrition* 19, 137S-146S.
- Hirayama, K., Rafter, J., 1999. The role of lactic acid bacteria in colon cancer prevention: mechanistic considerations. *Antonie van Leeuwenhoek* 76, 391 – 394.
- Hofmann, A. F., 1994. Bile acids. In: Arias, I.M., Boyer, J.L., Fausto, N., Jackoby, W.B. Schachter, D.A., Shafritz, D.A. (Eds.), *The liver: biology and pathobiology*, Shafritz Raven Press, New York, pp. 677–718.

- Hokanson, J.E., Austin, M.A., 1996. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level, a meta-analysis of population-based prospective studies. *Journal of Cardiovascular Risk* 3, 213-219.
- Höltzel, A., Gänzle, M.G., Nicolson, G.J., Hammes, W.P., Jung, G., 2000. The first low molecular weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. *Angewandte Chemie International Edition* 39, 2766-2768.
- Hoyos, A.B., 1999. Reduced incidence of necrotizing enterocolitis associated with enteral administration of *Lactobacillus acidophilus* and *Bifidobacterium infantis* to neonates in an intensive care unit. *International Journal of Disease* 3, 197-202.
- Huebner, J., Wang, Y., Krueger, W.A., Madoff, L.C., Martirosian, G., Biosot, S., Goldmann, D.A., Kasper, D.L., Tzianabos, A.O., Pier, G.B., 1999. Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of *Enterococcus faecalis* and vancomycin-resistant *Enterococcus faecium*. *Infection and Immunity* 67, 1213-1219.
- Husni, R.N., Gordon, S.M., Washington, J.A., Longworth, D.L., 1997. *Lactobacillus* bacteremia and endocarditis: review of 45 cases. *Clinical Infectious Diseases* 25, 1048-1055.
- Huttenen, E., Noro, k., Yang, Z., 1995. Purification and identification of antimicrobial substances produced by two *Lactobacillus casei* strains. *International Dairy Journal* 5, 503-513.
- Huyke, M.M., Spiegel, C.A., Gilmore, M.S., 1991. Bacteremia caused by hemolytic, high-level gentamycin-resistant *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy* 35, 1626-1634.
- Hynonen, U., Westerlund-Wikstrom, B., Palva, A., Korhonen, T.K. 2002. Fibronectin-binding function in the slpA surface protein of *Lactobacillus brevis*. *Journal of Bacteriology* 184, 3360-3367.
- Ike, Y., Hashimoto, H., Clewell, D.B., 1984. Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. *Infection and Immunity* 45, 528-530.
- Ike, Y., Hashimoto, H., Clewell, D.B., 1987. High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. *Journal of Clinical Microbiology* 25, 1524-1528.
- Ishikawa, H., Imaoka, A., Umesaki, Y., 2000. Randomized controlled trial of the effect of bifidobacterium-fermented milk on ulcerative colitis. *Gastroenterology* 118, (Suppl. 4), 779.

- Isolauri, E., Juntunen, M., Rautanen, T., Sillanauke, P., Koivula, T., 1991. A human *Lactobacillus* strain (*Lactobacillus* GG) promotes recovery from acute diarrhoea in children. *Pediatrics* 88, 90-97.
- Isolauri, E., Kaila, M., Mykkänen, H., Ling, W.H., Salminen, S., 1994. Oral bacteriotherapy for viral gastroenteritis. *Digestive and Disease Sciences* 39, 2595-2600.
- Isolauri, E., Arvola, T., Sütas, Y., Salminen, S., 2000. Probiotics in the management of atopic eczema. *Clinical Experimental Allergy* 30, 1605–1610.
- Isolauri, E., 2001. Probiotics in human disease. *American Journal of Clinical. Nutrition* 73, 6, 1142S-1146S.
- Isolauri, E., Sütas, Y., Kankaanpää, Arvilommi, H., Salminen, S., 2001. Probiotics: effects on immunity. *American Journal of Clinical Nutrition* 73, 444S-450S.
- Jack, R.W., Sahl, H.G., 1995. Unique peptide modifications involved in the biosynthesis of lantibiotics. *Trends in Biotechnology* 13, 269-278.
- Jack, R.W., Tagg, J.R., Ray, B., 1994. Bacteriocins of Gram-positive bacteria. *Microbiology Reviews* 59, 171–200.
- Jacobsen, C. N., Rosenfeldt-Nielsen, V., Hayford, A. E., Moller, P. L., Michaelsen, K. F., Paerregaard, A., Sandstrom, B., Tvede, M., Jakobsen, M., 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology* 65, 4949-4956.
- Jakava-Viljanen, M., Åvall-Jääskeläinen, S., Messner, S., Messner, P., Sleytr, U.B., Palva, A., 2002. Isolation of three new surface (S-) layer protein genes (*slp*) from *Lactobacillus brevis* ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions. *Journal of Bacteriology* 184, 6786–6795.
- Jay, J.M., 1982. Antimicrobial properties of diacetyl. *Applied and Environmental Microbiology* 44, 525-532.
- Jett, B.D., Jensen, H.G., Nordquist, R.E., Gilmore, M.S., 1992. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. *Infection and Immunity* 60, 2445-2452.
- Jett, B.D., Huycke, M.M., Gilmore, M.S., 1994. Virulence of enterococci. *Clinical Microbiology Reviews* 7, 462-478.

- Jiang, H.Q., Thurnheer, M.C., Zuercher, A.W., Boiko, N.V., Bos, N.A. and Cebra, J.J., 2004. Interactions of commensal gut microbes with subsets of B- and T-cells in the murine host. *Vaccine* 22, 805-811.
- Jin, L.Z., Marquardt, R.R., Zhao, X., 2000a. A strain of *Enterococcus faecium* (18C23) inhibits adhesion of enterotoxigenic *Escherichia coli* K88 to porcine small intestine mucus. *Applied and Environmental Microbiology* 66, 4200-4204.
- Jin, L.Z., Marquardt, R., Baidoo, S.K., Frohlich, A.A., 2000b. Characterization and purification of porcine small intestine mucus receptor for *Escherichia coli* K88ac fimbrial adhesion. *FEMS Immunology and Medical Microbiology* 27, 17-22.
- Johansson, M.L., Molin, G., Jeppson, B., Nobaek, S., Ahrne', S., Bengmark, S., 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup. *In vivo* colonization of human intestinal mucosa and effect on the indigenous flora. *Applied and Environmental Microbiology* 59, 15-20.
- Johnson, A.P., 1994. The pathogenicity of enterococci. *Journal of Antimicrobial Chemotherapy* 33, 1083-1089.
- Jones, J., Boorman, J., Cann, P., 2000. British Society of Gastroenterology guidelines for the management of the irritable bowel syndrome. *Gut* 47 (Suppl. 2), 1-19.
- Jonsson, H.; Ström, E., Roos, S., 2001. Addition of mucin to the growth medium triggers mucus-binding activity in different strains of *Lactobacillus reuteri* *in vitro*. *FEMS Microbiology Letters* 204, 19-22.
- Kabir, A.M., Aiba, Y., Takagi, A., Kamiya, S., Miwa, T., Koga, Y., 1997. Prevention of *Helicobacter Pylori* infection by lactobacilli in a gnotobiotic murine model. *Gut* 41, 49-55.
- Kadurugamuwa, J.L., Sin, L.V., Yu, J., Francis, K.P., Kimura, R., Purchio, T., Contag, P.R., 2003. Rapid direct method for monitoring antibiotics in a mouse model of bacterial biofilm infection. *Antimicrobial Agents in Chemotherapy* 47, 3130-3137.
- Kaila, M., Isolauri, E., Soppi, E., Virtanen, E., Laine, S., Arvilommi, H., 1992. Enhancement of the circulating antibody secreting cell response in human diarrhoea by a human *Lactobacillus* strain. *Pediatric Research* 32, 141-144.
- Kalliomaki, M., Salminen, S., Arvilommi, H., Pentti, K., Pertti, K., Isolauri, E., 2001. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 357, 1076-1079.

- Kamath, S.F., Phillips, S.F., O'Connor, M.K., Brown, M.L., Zinsmeister, A.R., 1990. Colonic capacitance and transit in man: modulation by luminal contents and drugs. *Gut* 31, 443–449.
- Kanazawa, H., Nagino, M., Kamiya, S., Komatsu, S., Mayumi, T., Takagi, K., Asahara, T., Nomoto, K., Tanaka, R. and Nimura, Y., 2005. Synbiotics reduce postoperative infectious complications: a randomized controlled trial in biliary cancer patients undergoing hepatectomy. *Langenbecks Archives of Surgery* 390, 104-113.
- Kankaanpää, P.E., Salminen, S.J., Isolauri, E., Lee, Y.K., 2001. The influence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiology Letters* 194,149-153.
- Katelaris, P.H., Salam, I., Farthing, M.J.G., 1995. Lactobacilli to prevent traveler's diarrhoea. *The Northern England Journal of Medicine* 333, 1360-1361.
- Kaur, I.P., Chopra, K., Saini, A., 2002. Probiotics: potential pharmaceutical applications. *European Journal of Pharmacology* 15, 1-9.
- Kawamoto, S., Shima, J., Sato, R., Educhi, T., Ohmomo, S., Shibato, J., Horikoshi, N., Takeshita, K., Sameshima, T., 2002. Biochemical and genetic characterization of mundticin KS and antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. *Applied and Environmental Microbiology* 68, 3830-3840.
- Kawase, M., Hashimoto, H., Hosoda, M., Morita, H., Hosono, A., 2000. Effect of administration of fermented milk containing whey protein concentrate to rats and healthy men on serum lipids and blood pressure. *Journal of Dairy Science* 83, 255–263.
- Kennedy, R.J., Hoper, M., Weir, H., 2000. Probiotic therapy stabilizes the gut mucosal barrier in the IL10 knockout mouse model of ulcerative colitis (abstract). *British Journal of Surgery* 87, 699.
- Kim, H.J., Camilleri, M., Mckinzie, S., Lempke, M.B., Burton, D.D., Thomforde, G.M., 2003. A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. *Alimentary Pharmacology and Therapeutics* 17, 895-904.
- Kim, H.J., Vazquez Roque, M.I., Camilleri, M., Stephens, D., Burton, D.D., Baxter, K., 2005. A randomized controlled trial of a probiotic combination VSL#3 and placebo in irritable bowel syndrome with bloating. *Neurogastroenterology and Motility* 17, 687-696.
- King, A.D.J., nagel, C.W., 1975. Influence of carbon dioxide upon the metabolism of *Pseudomonas aeruginosa*. *Journal of Food Science* 40, 362-366.

- Kirjavainen, P., Ouwehand, A., Isolauri, E., Salminen, S., 1998. The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters* 167, 185-189.
- Kishi, A., Kazuko, U., Matsubara, Y., Okuda, O., Kishida, T., 1996. Effect of the oral administration of *Lactobacillus brevis* subsp. *coagulans* on interferon- $\alpha$  producing capacity in humans. *Journal of the American College of Nutrition* 15, 408–412.
- Klaenhammer, T.R., 1988. Bacteriocins of lactic acid bacteria. *Biochimie* 70, 337–349.
- Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews* 12, 39-86.
- Klaver, F.A.M., van der Meer, R., 1993. The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium bifidum* is due to their bile salt-deconjugating activity. *Applied and Environmental Microbiology* 59, 1120-1124.
- Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W.E.J., Stiekema, W., Lankhorst, R.M.K., Bron, P.A., Hoffer, S.M., Groot, M.N.N., Kerkhoven, R. de Vries, M., Ursing, B., de Vos, W.M. and Siezen, R.J., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Sciences of the United States of America* 100, 4, 1990-1995.
- Korpela, R., Moilanen, E., Saxelin, M., Vapaasalo, H., 1997. *Lactobacillus rhamnosus* GG (ATCC 53013) and platelet aggregation *in vitro*. *International Journal of Food Microbiology* 37, 83–86.
- Kopp-Hoolihan, L., 2001. Prophylactic and therapeutic uses of probiotics: A review. *Journal of American Dietician Association* 101, 229 – 237.
- Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., Matošić, S., 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology* 94, 981–987.
- Kruis, W.; Schütz, E., Fric, P., 1997. Double-blind comparison of an oral *Escherichia coli* preparation and mesalamine in maintaining remission of ulcerative colitis. *Alimentary Pharmacology and Therapeutics* 11, 853–858.
- Kruis, W.; Kalk, E.V., Fric, P., 2001. Maintenance of remission in ulcerative colitis is equally effective with *Escherichia coli* Nissle 1917 and with standard mesalamine. *Gastroenterology* 120, (Suppl. 5), 127.

- Kühnen, E., Sahl, H.-G., Brandis, H., 1985. Purification and properties of LIQ 4, an antibacterial substance produced by *Streptococcus faecalis* var. *liquefaciens* K4. Journal of General Applied Microbiology 131, 1925-1932.
- Kukkonen, K., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., Tuure, T., 2007. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. Journal of Allergy and Clinical Immunology 119, 192–197.
- Kullen, M.J., Klaenhammer, T.R., 1999. Identification of the pH-inducible, proton-translocating F1F0-ATPase (atpBEFHAGDC) operon of *Lactobacillus acidophilus* by differential display: gene structure, cloning and characterization. Molecular Microbiology 33, 1152-1161.
- Landman, D., Quale, J.M., 1997. Management of infections due to resistant enterococci: a review of therapeutic options. Journal of Antimicrobial Chemotherapy 40, 161– 170.
- Lankaputhra, W.E.V., Shah, N.P., 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp in the presence of acid and bile salts. Cultured Dairy Products Journal 30, 2–7.
- Larsen, L.A., Raben, A., Haulrik, N., Hansen, A.S., Manders, M., Astrop, A., 2000. Effect of 8 week intake of probiotic milk products on risk factors for cardiovascular diseases. European Journal of Clinical Nutrition 54, 288-297.
- Larsen, N., Nissen, P., Willats, W.G.T., 2007. The effect of calcium ions on adhesion and competitive exclusion of *Lactobacillus* ssp. and *E. coli* O138. International Journal of Food Microbiology 114, 113-119.
- Lee, Y.-K., Puong, K.-Y., 2002. Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. British Journal of Nutrition 88 (Suppl.1), S101-S108.
- Lee, Y.-K., Puong, K.-Y., Ouwehand, A.C., Salminen, S., 2003. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. Journal of Medical Microbiology 52, 925-930.
- Lesuffleur, T., Barbat, A., Dussaulx, E., Zweibaum, A., 1990. Growth adaption to methotrexate of HT-29 human colon carcinoma cell is associated with their ability to differentiate into columnar absorptive and mucus secreting cells. Cancer Research 50, 6334-6343.
- Leclercq, R., 1997. Enterococci acquire new kinds of resistance. Clinical Infectious Diseases 24 (Suppl. 1), S80– S84.



- Lemoigne, M., 1927. Sur le métabolisme du diacétyle. CR Society of Biology 97, 1479-1481.
- Lewenstein, A., Frigerio, G., Moroni, M., 1979. Biological properties of SF68, a new approach for the treatment of diarrhoeal diseases. Current Therapeutic Research 26, 967-981.
- Lidbeck, A., Gustafsson, J.-Å., Nord, C.E., 1987. Impact of *Lactobacillus acidophilus* supplements on the human oropharyngeal and intestinal microflora. Scandinavian Journal of Infectious Diseases 19, 531-537.
- Lidbeck, A., Edlund, C., Gustafsson, J.-Å., Kager, L., Nord, C.E., 1988. Impact of *Lactobacillus acidophilus* on the normal intestinal microflora after administration of two antimicrobial agents. Infection 16, 329-36.
- Lidbeck, A., Överik, E., Rafter, J., Nord, C.E., Gustafsson, J.-A., 1992. Effect of *Lactobacillus acidophilus* supplements on mutagen excretion in faeces and urine in humans. Microbial Ecology in Health and Disease 5, 59 – 67.
- Lim, B.-K., Mahendran, R., Lee, Y.-K., Bay, B.H., 2002. Chemopreventive effect of *Lactobacillus rhamnosus* on growth of a subcutaneously implanted bladder cancer cell line in the mouse. Japanese Journal of Cancer Research 93, 36-41.
- Lin, M.-Y., Chen, T.-W., 2000. Reduction of cholesterol by *Lactobacillus acidophilus* in culture broth. Journal of Food and Drug Analysis 8, 97-102.
- Lin, W.-H., Hwang, C.-F., Chen, L.-W., Tsen, H.-Y., 2006. Viable counts, characterisatic evaluation for commercial lactic acid bacteria products. Food Microbiology 23, 74-81.
- Linden, P.K., Miller, C.B., 1999. Vancomycin-resistant enterococci: the clinical effect of a common nosocomial pathogen. Diagnostic Microbiology and Infectious Disease 33, 113-120.
- Lindgren, S.E., Dobrogosz, W.J., 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Reviews 87, 149-163.
- Ling, W.H., Korpella, R., Mykkanen, H., Salminen, S., Hanninen, O., 1994. *Lactobacillus* strain GG supplementation decreases colonic hydrolytic and reductive enzyme activities in healthy female adults. Journal of Nutrition 124, 18-23.
- Lortal, S., Van Heijenoort, J., Gruber, K., Sleytr, U.B., 1992. S-layer of *Lactobacillus helveticus* ATCC 12046: isolation, chemical characterization and re-formation after extraction with lithium chloride. Journal of General Microbiology 138, 611-618.
- Low, Y.L., Jakubovics, N.S., Flatman, J.C., Jenkinson, H.F., Smith, A.W., 2003. Manganese-dependant regulation of the endocarditis associated virulence factor EfaA of *Enterococcus faecalis*. Journal of Medical Microbiology 52, 113-119.



- Lowe, A.M., Lambert, P.A., Smith, A.W., 1995. Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesions from some oral streptococci. *Infection and Immunity* 63, 703-706.
- Lu, H.-Z, Weng, X.-H., Li, H., Yin, Y.-K., Pang, M.-Y., Tang, Y.-W., 2002. *Enterococcus faecium*-related outbreak with molecular evidence of transmission from pigs to humans. *Journal of Clinical Microbiology* 40, 913-917.
- Ma, D.; Forsythe, P., Bienenstock, J., 2004. Live *Lactobacillus reuteri* is essential for the inhibitory effect on tumor necrosis alpha-induced interleukin-8 expression. *Infection and Immunity* 72, 5308–5314.
- Mack, D.R., Michail, S., Wei, S., McDougall, L., Hollingsworth, M.A. (1999). Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. *American Journal of Physiology* 276, G941-G950.
- Majamaa, H., Isolauri, E., 1997. Probiotics: a novel approach in the management of food allergy. *Journal of Allergy and Clinical Immunology* 99, 179–185.
- Malchow, H.A., 1997. Crohn's disease and *Escherichia coli*. A new approach in therapy to maintain remission of colonic Crohn's disease? *Journal of Clinical Gastroenterology* 25,, 653–658.
- Malin, M.; Suomalainen, H.; Saxelin, M., Isolauri, E., 1996. Promotion of IgA immune response in patients with Crohn's disease by oral bacteriotherapy with *Lactobacillus* GG. *Annals in Nutrition Methods* 40, 137–145.
- Mann, G.V., 1974. Studies of a surfactant and cholesterolemia in the Maasai. *American Journal of Clinical Nutrition* 27, 464-469.
- Marteau, P., Pochart, P., Flourié, B., Pellier, P., Santos, L., Desjeux, J.F.and Rambaud, J.C., 1990. Effect of chronic ingestion of a fermented dairy product containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on metabolic activities of the colonic flora in humans. *American Journal of Clinical Nutrition* 52, 685–688.
- Marteau, P., Seksik, P., Jian, R., 2002. Probiotics and health: new facts and ideas *Current Opinion in Biotechnology*, 13,, 486-489.
- Marteau, P., Shanahan, F., 2003. Basic aspects and pharmacology of probiotics: an overview of pharmacokinetics, mechanisms of action and side-effects. *Best Practice in Research and Clinical Gastroenterology* 17, 725-740.
- Martinez, F.D., Holt, P.G., 1999. Role of microbial burden in aetiology of allergy and asthma. *Lancet* 354, (Suppl. 2), 12–15.

- Martinez, B., Rodriguez, A., Suarez, J.E., 2000. Lactococcin 972, a bacteriocin that inhibits septum formation in lactococci. *Microbiology* 146, 949-955.
- Mason, D.J., Shanmuganathan, S., Mortimer, F.C., Gant, V.A., 1998. A fluorescent Gram stain for flow cytometry and epifluorescence microscopy. *Applied and Environmental Microbiology*. 64, 2681-2685.
- Masuda, K., Kawata, T., 1981. Characterization of a regular array in the wall of *Lactobacillus buchneri* and its reattachment to the other wall components. *Journal of General Microbiology* 124, 81-90.
- Masuda, K., Kawata, T., 1983. Distribution and chemical chracterization of regular arrays in the cell walls of strains of the genus *Lactobacillus*. *FEMS Microbiology Letters* 20, 145-150.
- Masuda, K., 1992. Heterogeneity of S-layer proteins of *Lactobacillus acidophilus* strains. *Microbial Immunology* 36, 297-301.
- Masuoka, J.C., Hazen, K., 1999. Differences in the acid-labile component of *Candida albicans* mannan from hydrophobic and hydrophilic yeast. *Glycobiology* 9, 1281-1286.
- Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria- a review. *International Journal of Food Microbiology* 105, 281-295.
- Matsumoto, S., Watanabe, N., Imaoka, A., Okabe, Y., 2001. Preventative effects of *Bifidobacterium*- and *Lactobacillus*-fermented milk on the development of inflammatory bowel disease in senescence-accelerated mouse P1/Yit strain mice. *Digestion*, 64, 92-99.
- Matsumura, A.T., Saito, T., Arakuni, M., Kitazawa, H., Kawai, Y., Itoh, T., 1999. New binding assay and preparative trial of cell-surface lectin from *Lactobacillus acidophilus* group lactic acid bacteria. *Journal of Dairy Science* 82, 2525-2529.
- McAuliffe, O., Ross, R.P., Hill, C., 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews* 25, 285-308.
- McCarthy, J., O'Mahony, L., O'Callaghan, L., Sheil, B., Vaughan, E.E., Fitzsimmons, N., 2003. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut* 52, 975-980.
- McGroarty, J.A., Reid, G., 1988. Detection of a *Lactobacillus* substance which inhibits *Escherichia coli*. *Canadian Journal of Microbiology* 34, 974-978.
- McIntosh, G.H., 1996. Probiotics and colon cancer prevention. *Asia Pacific Journal of Clinical Nutrition* 271, 1913-1918.

- Menard, S., Candalh, C., Bambou, J.C., Terpend, K., Cerf-Bensussan, N., Heyman, M., 2004. Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. *Gut* 53, 821–828.
- Metchnikoff, E., 1907. *The prolongation of life*, London: William Heinemann.
- Michetti, P., Dorta, G., Wiesel, P.H., Brassart, D., Verdu, E., Herranz, M., Felley, C., Porta, N., Rouvet, M., Blum, A.L., Corthesy-Theulaz, I., 1999. Effect of whey-based culture supernatant of *Lactobacillus acidophilus (johnsonni)* La1 on *Helicobacter pylori* infection in humans. *Digestion* 60, 203-209.
- Midvedt, A.C., Carlstedt-Duke, B., Midvedt, T., 1994. Establishment of a mucin-degrading intestinal microflora during the first two years of life. *Journal of Pediatric Gastroenterology and Nutrition* 18, 321-326.
- Miettinen, M., Vuopio-Varkila, J., Varkila, K., 1996. Production of human necrosis factor  $\alpha$ , interleukin 6, and interleukin 10 is induced by lactic acid bacteria. *Infection and Immunity* 64, 5403–5405.
- Millar, M.R., Bacon, C., Smith, S.L., Walker, V., Hall, M.A., 1993. Enteral feeding of premature infants with *Lactobacillus* sp. strain GG. *Archives of Disease in Childhood* 69, 483-487.
- Mimura, P., Rizzello, F., Schreiber, S., 2002. Once daily high dose probiotic therapy maintains remission and improved quality of life in patients with recurrent or refractory pouchitis: a randomized, placebo-controlled, double-blind trial. *Gastroenterology* 122, 81.
- Miraglia del Giudice, M., Papasso, M., Macello, N., Capristo, C., Piacentini, G.L., Brunese, F.P., 2003. Exhaled nitric oxide and atopy in children. *Journal of Allergy and Clinical Immunology* 11, 193–198.
- Mishra, V., Prasad, D.N., 2005. Application of *in vitro* methods for selection of *Lactobacillus casei* 376 strains as potential probiotics. *International Journal of Food Microbiology* 15, 109-115.
- Miyazaki, S., Ohno, A., Kobayashi, I., Yamaguchi, K., Goto, S., 1993. Cytotoxic effect of haemolytic culture supernatant from *Enterococcus faecalis* on mouse polymorphonuclear neutrophils and macrophages. *Microbiology and Immunology* 37, 265-270.
- Moellering, R.C., 1990. The enterococci: An enigma and a continuing therapeutic challenge. *European Journal of Clinical Microbial and Infectious Diseases* 9, 73–74.
- Moellering, R.C., 1991. The *Enterococcus*: a classic example of the impact of antimicrobial resistance on therapeutic options. *Antimicrobial Chemotherapy* 28, 1–12.

- Moellering, R.C., 1992. Emergence of *Enterococcus* as a significant pathogen. *Clinical Infection and Disease* 14, 1173–1178.
- Montes, R.G., Bayless, T.M, Saavedra, J.M., Perman, J.A., 1995. Effect of milks inoculated with *Lactobacillus acidophilus* or a yoghurt started culture in lactose-maldigesting children. *Journal of Dairy Science* 78, 1657 – 1664.
- Morotomi, M., Guillem, J.G., LoGerfo, P., Weinsten, I.B., 1990. Production of diacylglycerol, an activator of protein kinase C by human intestinal microflora. *Cancer Research* 50, 3595-3599.
- Morris, J.G., 1976. Oxygen and the obligate anaerobe. *Journal of Applied Bacteriology* 40, 229-244.
- Morrison, D., Woodford, N., Cookson, B., 1997. Enterococci as emerging pathogens of humans. *Journal of Applied Microbiology Symposium Supplement*. 83, 89S– 99S.
- Moser, S.A.and Savage, D.C., 2001. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Applied and Environmental Microbiology* 67, 3476–3480.
- Mundy, L.M., Sahm, D.F., Gilmoie, M., 2000. Relationships between enterococcal virulence and antimicrobial resistance. *Clinical Microbiology Reviews* 13, 513-522.
- Murray, B.E., 1990. The life and times of the enterococcus. *Clinical Microbiology Reviews* 3, 46–65.
- Nagao, F., Nakayama, M., Muto, T., Okomura, K., 2000. Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the immune system in healthy human subjects. *Bioscience Biotechnology and Biochemistry* 64, 2706–2708.
- Nallaparreddy, S.R., Qin, X., Weinstock, G.M, Höök, M., Murray, B.E., 2000a. *Enterococcus faecalis* adhesion, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infection and Immunity* 68, 5218-5224.
- Nallaparreddy, S.R., Singh, K.V., Duh, R.W., Weinstock, G.M., Murray, B.E., 2000b. Diversity of ace, a gene encoding a microbial surface component recognizing adhesive matrix molecules, from different strains of *Enterococcus faecalis* and evidence for production of ace during human infections. *Infection and Immunity* 68, 5210-5217.
- Nallaparreddy, S.R., Weinstock, G.M., Murray, B.E., 2003. Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acn, a new member of the MSCRAMM family. *Molecular Microbiology* 47, 1733-1747.

- Neeser, J.R., Granato, D., Rouvet, M., Servin, A., Teneberg, S., Karlsson, K.A., 2000. *Lactobacillus johnsonii* La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria. *Glycobiology* 10, 1193-1199.
- Nes, I.F., Diep, D.B., Håverstein, L.S., Brurberg, M.B., Eijsink, V., Holo, H., 1996. Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek* 70, 113-128.
- Nguyen, T.D.T., Kang, J.H., Lee, M.S., 2007. Characterization of a potential probiotic bacterium *Lactobacillus fermentum* PH04 with cholesterol lowering-effect. *International Journal of Food Microbiology* 113, 356-361.
- Nilsen, T., Nes, I.F., Holo, H., 2003. Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. *Applied and Environmental Microbiology* 69, 2975-2984.
- Nomoto, K., 2005. Review: Prevention of infections by probiotics. *Journal of Bioscience and Bioengineering* 100, 583-592.
- Norin, K.E., Carlstedt-Duke, B., Hoverstad, T., Lingaas, E., Saxerholt, H., Steinbakk, M., Midvedt, T., 1988. Faecal tryptic activity in humans influence of antibiotics on microbial intestinal degradation. *Microbial and Ecology in Health Disease* 1, 65.
- Oakley, H.J., Harty, D.W.S., Knox, K.W., 1995. Enzyme production by lactobacilli and the potential link with infective endocarditis. *Journal of Applied Bacteriology* 78, 142-148.
- Oberhelman, R.A., Gilman, R.H., Sheen, P., 1999. A placebo-controlled trial of *Lactobacillus* GG to prevent diarrhoea in undernourished Peruvian children. *Journal of Pediatrics* 134, 15-20.
- Okkers, D.J., Dicks, L.M.T., Silvester, M., Joubert, J.J., Odendaal, H.J., 1999. Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with fungistatic effect on *Candida albicans*. *Journal of Applied Microbiology* 87, 726-734.
- Oksanen, P.J., Salminen, S., Saxelin, M., Hamalainen, P., Ihanola-Vormisto, A., Muurasniemi-Isoviita, L., Nikkari, S., Oksanen, T., Porsti, I., Salminen, E., 1990. Prevention of traveler's diarrhoea by *Lactobacillus* GG. *Annals in Medicine* 22, 53-56.
- Olden, K., 2002. Diagnosis of irritable bowel syndrome. *Gastroenterology* 122, 1701-1714.
- O'Mahony, L., Feeney, M., O'Halloran, S., Murphy, L., Kiely, B., Fitzgibbon, J., Lee, G.O., Sullivan, G., Shanahan, F., Collins, J.K., 2001. Probiotic impact on microbial flora, inflammation and tumour development in IL10 knockout mice. *Alimentary Pharmaceutical Therapy* 15, 1219-1225.

- O'Mahony, L., McCarthy, J., Kelly, P., Hurley, G., Luo, F., Chen, K., 2005. *Lactobacillus* and *Bifidobacterium* in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 128, 541–551.
- Orrhage, K., Brismar, B., Nord, C.E., 1994. Effect of supplements with *Bifidobacterium longum* and *Lactobacillus acidophilus* on the intestinal microbiota during administration of clindamycin. *Microbial Ecology in Health and Disease* 7, 17–25.
- Orrhage, K., Sillerstrom, E., Gustafsson, J.-A., Nord, C.E., Rafter, J., 1994. Binding of mutagenic heterocyclic amines by intestinal and lactic acid bacteria. *Mutation Research* 311, 239–248.
- Ouwehand, A.C., Kirjavainen, P.V., Grönland, M.-M., Isolauri, E., Salminen, S.J., 1999. Adhesion of probiotic micro-organisms to intestinal mucus. *International Dairy Journal* 9, 623-630.
- Ouwehand, A.C., Tuomola, E.M., Tölkö, S., Salminen, S., 2001. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. *International Journal of Food Microbiology* 64, 119-126.
- Ouwehand, A.C., Salminen, S., Roberts, P.J., Ovaska, J., Salminen, E., 2003. Disease-dependent adhesion of lactic acid bacteria to the human intestinal mucosa. *Clinical and Diagnostic Laboratory Immunology*, 10, 643-646.
- Ouwehand, A.C., Vesterlund, S., 2004. Antimicrobial components from lactic acid bacteria. In: Salminen, S., von Wright, Ouwehand, A., (Eds.), *Lactic acid bacteria, Microbiological and Functional aspects*, Marcel Dekker, Inc., pp. 375-395.
- Padan, E., Zilberstein, D., Schuldiner, S., 1981. pH homeostasis in bacteria. *Biochimica et Biophysica Acta* 650, 151-166.
- Papadelli, M., Manolopoulou, E., Kalantzopoulos, G., Tsakalidou, E. (2003). Rapid detection and identification of *Streptococcus macedonicus* by species-specific PCR and DNA hybridisation. *International Journal of Food Microbiology* 81, 231-239.
- Parvez, S., Malik, K.A., Khang, A., Kim, H.Y., 2006. Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology* 100, 1171-1185.
- Pathmakanthan, S.; Li, C. Cowie, J., Hawkey, C.J. (2004). *Lactobacillus plantarum* 299: Beneficial *in vitro* immunomodulation in cells extracted from inflamed human colon. *Journal of Gastroenterology and Hepatology* 19, 166–173.

- Pelletier, C., Bouley, C., Bourliov, P., Carbon, C., 1996. Evaluation of safety properties of *Lactobacillus* strains by using an experimental model of endocarditis in rabbit. Ab-SOMED Meeting, Paris, 1996.
- Pennacchia, C., Vaughan, E.E., Villani, F., 2006. Potential probiotic *Lactobacillus* strains from fermented sausages: Further investigations on their probiotic properties. Meat Science 73, 90-101.
- Perdigon, G., De Jorrat, M.E.B., De Petrino, S.F., De Budeguer, M.V., 1991. Effect of oral administration of *Lactobacillus casei* on various biological functions of the host. Food Agricultural and Immunology 3, 93–102.
- Pereira, D.I.A., Gibson, G.R., 2002. Effects of consumption of probiotics and prebiotics on serum blood levels of humans. Critical Reviews in Biochemistry and Molecular Biology 37 , 259-281.
- Perez, P.F., Minnaard, J., Rouvet, M. 2001. Inhibition of *Giardia intestinalis* by extracellular factors from *Lactobacilli*: an *in vitro* study. Applied and Environmental Microbiology 67, 5037-5042.
- Pessi, T.; Sutas, Y., Hurme, M., Isolauri, E., 2000. Interleukin-10 generation in atopic children following oral *Lactobacillus rhamnosus* GG. Clinical Experimental Allergy 30, 1804–1808.
- Pfeifer, A., Rosat, J-P., 1999. Probiotics in alimentation: Clinical evidence for their enhancement of the natural immunity of the gut. In: Hanson, L.A. and Robert, H.Y., (Eds.), Probiotics, other nutritional factors and intestinal microflora, vol. 42, Nestlé Nutrition Workshop Series, Nestec Ltd., Philadelphia: Vevey/Lillincott-Raven Publishers, pp. 244-257.
- Piard, J.C., Desmazeaud, M., 1992a. Inhibiting factors produced by lactic acid bacteria: I. Oxygen metabolites and catabolism end-products. Lait 71, 525–541.
- Piard, J.C., Desmazeaud, M., 1992b. Inhibiting factors produced by lactic acid bacteria: II. Bacteriocins and other antimicrobial substances. Lait 72, 113–142.
- Pinto M., Robine-Leon, S., Appay, M.D., Kedinger, M., Tiadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Hafen, K., Fogh, J., Zweibaum, A. 1983. Enterocyte like differentiation of cultured human colon carcinoma cell line Caco-2 in culture. Biology of the Cell 47, 323-330.
- Pinto, M.G.V., Franz, M.A.P., Schillinger, U., Holzapfel, W.H. 2006. *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. International Journal of Food Microbiology 109, 205-214.



- Pohjavuori, E., Viljanen, M., Korpela, R., Kuitunen, M., Tiittanen, M., Vaarala, O., 2004. *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy. *Journal of Allergy and Clinical Immunology* 114, 131–136.
- Poole, K., 2002. Mechanisms of bacterial biocide and antibiotic resistance. *Journal of Applied Microbiology* 92 (Suppl.1), 55S– 64S.
- Pool-Zobel, B., Bertram, B., Knoll, M., Lambertz, R., Neudecker, C., Schillinger, U., Schmezer, P., Holzapfel, W.H., 1993. Antigenotoxic properties of lactic acid bacteria *in vivo* in the GI tract of rats. *Nutrition and Cancer* 20, 271–281.
- Pool-Zobel, B.L., Neudecker, C., Domizlaff, I., Ji, S., Schillinger, U., Rumney, C., Moretti, M., Vilarini, I., Scassellati-Sforzolini, R., Rowland, I., 1996. *Lactobacillus* and *Bifidobacterium* mediated antigenotoxicity in the colon of rats. *Nutrition and Cancer* 26, 365–380.
- Prantera, C., Scribano, M.L., Falasco, G., 2002. Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomized controlled trial with *Lactobacillus* GG. *Gut* 51, 405–409.
- Prescott, S.L., Dunstan, J.A., Hale, J., Breckler, L., Lehmann, H., Weston, S., 2005. Clinical effects of probiotics are associated with increased interferon-gamma responses in very young children with atopic dermatitis. *Clinical and Experimental Allergy* 35, 1557–1564.
- Prescott, S.L., Björkstén, B., 2007. Probiotics for the prevention and treatment of allergic diseases. *Current Perspectives*, in press.
- Pridmore, R.D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A.-C., Zwahlen, M.-C., Rouvet, M., Altermann, E., Barrangou, R., Mollet, B., Mercenier, A., Klaenhammer, T., Arigoni, F., Schnell, M.A., 2004. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proceedings of the National Academy of Science of the United States of America*. 101, 2512-2517.
- Pultz, N.J., Versturland, S., Ouwehand, A.C., Donskey, C.J., 2006. Adhesion of vancomycin-resistant *Enterococcus* to human intestinal mucus. *Current Microbiology* 52, 221-224.
- Qin, X, Singh, K.V., Weinstock, G.M., Murray, B.E., 2000. Effects of *Enterococcus faecalis* *fsr* genes on production of gelatinase and a serine protease and virulence. *Infection and Immunity* 68, 2579-2586.
- Rakita, R.M., Vanek, N.N., Jaquez-Palas, K., Mee, M., Mariscalco, M.M., Dunny, G.M., Snuggs, M., van Winkle, W.B., Simon, S.I., 1999. *Enterococcus faecalis* bearing aggregation



- substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infection and Immunity* 67, 6067-6075.
- Rastall, R.A., Gibson, G.R., 2002. Prebiotic oligosaccharides: Evaluation of biological activities and potential future developments. In: Tannock, G.W., (Ed.), *Probiotics and Prebiotics: Where are we going?*, Caister Academic Press, Norfolk, England, pp.107 – 147.
- Rayes, N., Seehofer, D., Hansen, S., Boucsein, K., Muller, A.R., Serke, S., Bengmark, S., Neuhaus, P., 2002a. Early enteral supply of *Lactobacillus* and fiber versus selective bowel decontamination; a controlled trial in liver transplant recipients. *Transplantation* 74, 123-127.
- Rayes, N., Hansen, S., Seehofer, D., Boucsein, K., Muller, A.R., Serke, S., Bengmark, S. and Neuhaus, P., 2002b. Early enteral supply of fibre and lactobacilli versus conventional nutrition: a controlled trial in patients with major abdominal surgery. *Nutrition* 18, 609-615.
- Reid, G., Cook, R.L., Bruce, A.W., 1987. Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. *Journal of Urology* 138, 330-335.
- Reid, G., Bruce, A.W., 1995. Low vaginal pH and urinary-tract infection. *Lancet* 346, 1704.
- Reid, G., Bruce, A.W., Taylor, M., 1995. Instillation of *Lactobacillus* and stimulation of indigenous organisms to prevent recurrence of urinary tract infections. *Microecology and Therapy* 23, 32-45.
- Reid, G., Bruce, A.W., 2001. Selection of *Lactobacillus* strains for urogenital probiotic applications. *Journal of Infection and Diseases* 183, S77-S80.
- Reid, G., Howard, J., Gan, B.S., 2001. Can bacterial interference prevent infection? *Trends in Microbiology* 9, 424-428.
- Reid, G., Burton, J., 2002. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbiology and Infection* 4, 319-324.
- Reid, G., Charbonneau, D., Erb, J., Kochanowski, B., Beuerman, D., Poehner, R., Bruce, A.W., 2003. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunology and Medical Microbiology* 35, 131-134.
- Reid, G., Jass, J., Sebelsky, M.T., McCormick, J.K., 2003. Potential uses of probiotics in clinical practice. *Clinical and Microbiology Reviews* 16, 658-672.

- Rembacken, D.J., Snelling, A.M., Hawkey, P.M., 1999. Non-pathogenic *Escherichia coli* versus mesalamine for the treatment of ulcerative colitis: a randomized trial. *Lancet* 354, 635–639.
- Renner, H.W., Musner, R., 1991. The possible role of probiotics as dietary antimutagens. *Mutation Research* 262, 239–245.
- Rice, L.B., Carias, L., Rudin, S., Vael, C., Goossens, H., Konstabel, C., Klare, I., Nallapareddy, S.R., Huang, W., Murray, B.E., 2003. A potential virulence gene *hyl<sub>Efm</sub>*, predominates in *Enterococcus faecium* of clinical origin. *Journal of Infectious Diseases* 187, 508-512.
- Roberfroid, M.B., 2000. Prebiotics and probiotics: are they functional foods. *American Journal of Clinical Nutrition* 71 (Suppl), 1682S – 1687S.
- Rodríguez, J.M., Martínez, M.I., Horn, N., Dodd, H.M., 2003. Heterologous production of bacteriocins by lactic acid bacteria. *International Journal of Food Microbiology* 80, 101-116.
- Rolfe, R.D., 2000. The role of probiotic cultures in the control of gastro-intestinal health, *Journal of Nutrition* 130(2S), 396S-402S.
- Roos, S., Jonsson, H., 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology* 148, 433-442.
- Rossi, E.A., Vendramini, R.C., Carlos, I.Z., Pei, Y.C., de Valdez, G.F., 1999. Development of a novel fermented soymilk product with potential probiotic properties. *European Food Research and Technology* 209, 305-307.
- Rowland, I.R., 1988. Role of the gut microflora in toxicity and cancer. Academic Press, London.
- Rozdzinski, E., Marre, R., Susa, M.; Wirth, R., Muscholl-Silber-horn, A., 2001. Aggregation substance-mediated adherence of *Enterococcus faecalis* to immobilized extracellular matrix proteins. *Microbial Pathogens* 30, 211-220.
- Ruiz, P.A., Hoffmann, M., Szesny, S., Blaut, M., Haller, D., 2005. Innate mechanisms for *Bifidobacterium lactis* to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats. *Immunology* 115, 441-450.
- Saavedra, J.M., Frank, A., 2002. The immunological promises of probiotics and probiotics. ORAFI Newsletter Number 6 February 2002.
- Sadikot, R.T., Blackwell, T.S., 2005. Bioluminescence Imaging. *Proc. Am Thorac Soc* 2, 537-540.

- Sakamoto, I., Igarashi, M., Kimura, K., Takagi, A., Miwa, T., Koga, Y., 2001. Suppressive effect of *Lactobacilli gasseri* OLL 2716 (LG21) on *Helicobacter pylori* infection in humans. *Journal of Antimicrobial Chemotherapy* 47, 709-710.
- Salminen, S., Isolauri, E., Onnela, T., 1995. Gut flora in normal and disordered states. *Chemotherapy* 41, S5-S15.
- Salminen, S. Bouley, C., Boutron-Ruault, M.C., 1998a. Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition* 80 (Suppl. 1), 147–171.
- Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W.M., Fondén, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.E., Matilla-Sandholm, T., 1998b. Demonstration of safety of probiotics - a review. *International Journal of Food Microbiology* 44, 93-106.
- Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stamatii, A., Zucco, F., 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology* 21, 1-26.
- Sánchez-Hidalgo, M., Maqueda, M., Gálvez, A., Abriouel, H., Valdivia, E., Martínez-Bueno, M., 2003. The genes coding for enterocin EJ97 production by *Enterococcus faecalis* EJ97 are located on a conjugative plasmid. *Applied and Environmental Microbiology* 69, 1633-1641.
- Sanders, M.E., 1993. Effect of consumption of lactic acid cultures on human health. *Advances in Food and Nutrition Research* 37, 67-130.
- Sanfilippo, L., Li, C.K., Seth, R., Balwin, T.J., Menozzi, M.G., Mahida, Y.R., 2000. *Bacteroides fragilis* enterotoxin induces the expression of IL-8 and transforming growth factor-beta (TGF-beta) by human colonic epithelial cells. *Clinical Experimental Immunology* 119, 456–463.
- Sanford, P.A., 1992. *Digestive System and Physiology*, London: Edward Arnold.
- Sára, M., Sleytr, U.B., 2000. S-layer proteins. *Journal of Bacteriology* 182, 859-868.
- Sarem-Damerdj, L.-O., Sarem, F., Marcjal, L., Nicolas, J.-P., 1995. *In vitro* colonization ability of human colon mucosa by exogenous *Lactobacillus* strains. *FEMS Microbiology Letters* 131, 133-137.
- Satoh, E., Leer, R.J., Conway, P.L., Pouwels, P.H., 1999. Mucus adhesion promoting protein of *Lactobacillus reuteri* 104R: 6<sup>th</sup> Symposium on Lactic acid Bacteria, Veldhoven, the Netherlands, 9.

- Savage, D.C., 1992. Growth phase, cellular hydrophobicity, and adhesion *in vitro* of lactobacilli colonizing the keratinizing gastric epithelium in the mouse. *Applied and Environmental Microbiology* 58, 1992-1995.
- Saxelin, M., Elo, S., Salminen, S., Vapaatalo, H., 1991. Dose response colonization of faeces after oral administration of *Lactobacillus casei* strain GG. *Microbial Ecology in Health and Disease* 4, 209-214.
- Saxelin, M., Ahokas, M., Salminen, S., 1993. Dose response of the faecal colonization of *Lactobacillus* strain GG administered in two different formulations. *Microbial Ecology in Health and Disease* 6, 119-122.
- Saxelin, M., 1996. Colonization of the human gastro-intestinal by probiotic bacteria. *Nutrition Today* 31, 5S-8S.
- Saxelin, M., 1997. *Lactobacillus* GG - a human probiotic strain with thorough clinical documentation. *Food Reviews International* 13, 293-313.
- Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., de Vos, W.M., 2005. Probiotic and functional microbes: from markets to mechanisms. *Current Opinion in Biotechnology* 16, 204-211.
- Scaletsky, I.C.A., Fabbriotti, S.H., Aranda, K.R., Morais, M.B., Fagundes-Netos, U., 2002. Comparison of DNA hybridization and PCR assays for detection of putative pathogenic enteroadherent *Escherichia coli*. *Journal of Clinical Microbiology* 40, 1254-1258.
- Schaafsma, G., Meuling, W.J.A., van Dokkum, W., Bouley, C., 1998. Effects of a milk product, fermented by *Lactobacillus acidophilus* and with fructo-oligosaccharides added, on blood lipids in male volunteers. *European Journal of Clinical Nutrition* 52, 436-440.
- Schaarmann, G., Schneider, J., Zorn, A., Vilser, C., Jahreis, G., 2001. Influence of probiotic yoghurt on serum lipids in women. *American Journal of Clinical Nutrition* 73 (Suppl.), 496S.
- Schlegel, H.G., 1985. *Allgemeine Mikrobiologie*, 6<sup>th</sup> Ed., Georg Thieme Verlag: Stuttgart, pp. 571
- Schierack, P., Nordhoff, M., Pollmann, M.M, Weyrauch, K.D., Amasheh, S., Lodemann, U., Jores, J., Tachu, B., Kleta, S., Blikslager, A., Tedin, K., Wieler, L.H., 2005. Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine. *Histochem. Cell Biology* 125, 293-305.
- Schillinger, U., Guigas, C., Holzapfel, W.H., 2005. *In vitro* adherence and other properties of lactobacilli used in probiotic yoghurt-like products. *International Dairy Journal* 15, 1289-1297.

- Schmidt, E.J., Boswell, J.S., Walsh, J.P., Schellenberg, M.M., Winter, T.W., Li, C., Allman, C.W., Savage, P.V., 2001. Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria. *Journal of Antimicrobial Chemotherapy* 47, 671–674.
- Schmidtchen, A., Frick, I.-M., Andersson, E., Tapper, H., Björck, L., 2002. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Molecular Microbiology* 46, 157-168.
- Schneitz, C., Nuotio, L., Lounatma, K., 1983. Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). *Journal of Applied Bacteriology* 74, 290-294.
- Schwab, J.H., 1993. Phylogistic properties of peptide-glycan-polysaccharide polymers from cell walls of pathogenic and normal-flora bacteria which colonise humans. *Infection and Immunity* 61, 4535–4539.
- Sewankambo, N., Gray, R.H., Wawer, M.J., Paxton, L., McNaim, D., Wabwire-Mangen, F., Serwadda, D., Li, C., Kiwanuka, N., Hillier, S.L., 1997. HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet* 350, 546-550.
- Shanahan, F., 2001. Inflammatory bowel disease: immunodiagnostics, immunotherapeutics and ecotherapeutics. *Gastroenterology* 120, 622-635.
- Shanahan, F., 2002. Crohn's disease. *Lancet* 359, 62-69.
- Shanahan, F., 2003. Crohn's disease. *Science and Medicine* 9, 48-58.
- Shankar, V., Baghdayan, A.S., Huycke, M.M., Lindahl, G., Gilmore, M.S., 1999. Infection-derived *Enterococcus faecalis* strains are enriched in esp, a gene encoding a novel surface protein. *Infection and Immunity* 67, 193-200.
- Sharper, A.G., Jones, K.W., Jones, M., Kyobe, J., 1963. Serum lipids in three nomadic tribes of northern Kenya. *American Journal of Clinical Nutrition* 13, 135-146.
- Shermak, M.A., Saavedra, J.M., Jackson, T.L., Huang, S.S., Bayless, T.M., Perman, J.A., 1995. Effect of yogurt on symptoms and kinetics of hydrogen production in lactose-malabsorbing children. *American Journal of Clinical Nutrition* 62, 1003–1006.
- Shornikova, A.V.; Casas, I. A., Isolauri, E., Mykkanen, H., Vesikari, T., 1997. *Lactobacillus reuteri* as a therapeutic agent in acute diarrhoea in young children. *Journal of Pediatric Gastroenterology and Nutrition* 24, 399-404.
- Silanpää, J., Martinez, B., Antikainen, J., Toba, T., Kalkkinen, N., Tankka, S., Lounatmaa, K., Keränen, J., Höök, M., Westurland-Wikström, B., Pouwels, P.H., Korhonen, T.K.,

2000. Characterization of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. *Journal of Bacteriology* 182, 6446-6450.
- Simpson, W.J., Hammond, J.R.M., Miller, R.B., 1988. Avoparcin and vancomycin-Useful antibiotics for the isolation of brewery lactic acid bacteria. *Journal of Applied Bacteriology* 64, 299– 309.
- Singh, K.V., Qin, X., Weinstock, G.M., Murray, B.E., 1998. Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. *Journal of Infectious Diseases* 178, 1416-1420.
- Sleytr, U.B., Beveridge, T.J., 1999. Bacterial S-layers. *Trends in Microbiology* 7, 253–260.
- Slonczewski, J.L., Rosen, B.P., Alger, J.R., Macnab, R.M., 1981. pH homeostasis in *Escherichia coli*: measurement by <sup>31</sup>P nuclear magnetic resonance of methylphosphonate andphosphate. *Proceedings of the National Academy of Science in the United Stated of America* 78, 6271-6275.
- Spergel, J.M., Paller, A.S., 2003. Atopic dermatitis and the atopic march. *The Journal of Clinical Immunology* 112(Suppl.), S118-S127.
- Steidler, L., Hans, W. and Schotte, L., 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* 289, 1352–1355.
- Su, Y.A., Sulavik, M.C., He, P., Mäkinen, P., Fiedler, S., Wirth, R., Clewell, D.B., 1991. Nucleotide sequence of the gelatinase gene (gelE) from *Enterococcus faecalis* subsp. *liquefaciens*. *Infection and Immunity* 59, 415-420.
- Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C., Koga, Y., 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *Journal of Immunology* 159, 1739–1745.
- Sullivan, Å., Nord, C.E., 2002. The place of probiotics in human intestinal infections: Review. *International Journal of Antimicrobial Agents* 20, 313-319.
- Svennerholm, A.M., Quiding-Järbrink, M., 2003. Priming and expression of immune responses in the gastric mucosa. *Microbial Infections* 5, 731-739.
- Szajewska, H., Mrukowicz, J.Z., 2001. Probiotics in the treatment and prevention of acute infectious diarrhoea in infants and children: a systematic review of published randomized, double-blind, placebo-controlled trials. *Journal of Pediatric Gastroenterology and Nutrition* 33 (Suppl.2), S17-25.

- Szajewska, H., Kotowska, M., Mrukowicz, J.Z., Armanska, M., Mikotajczyk, W., 2001. Efficacy of *Lactobacillus* GG in prevention of nosocomial diarrhoea in infants. *Journal of Pediatric* 138, 361-365.
- Tagg, J.R. Dajani, A.S. Wannamaker, L.W., 1976. Bacteriocins of Gram-positive bacteria, *Microbiology Reviews* 722–756.
- Takagi, A., Takeshi, M., Sato, M., Nomoto, K., Morotomi, M., Yokokura, T., 2001. Enhancement of natural killer cell cytotoxicity delayed murine carcinogenesis by a probiotic microorganism. *Carcinogenesis* 22, 599–605.
- Tamboli, C.P., Caucheteux, C., Cortot, A., Colombel, J.-F., Desreumaux, P., 2003. Probiotics in inflammatory bowel disease: a critical review. *Best practice and Research in Clinical Gastroenterology* 17, 805-820.
- Tang, P., Foubister, V., Pucciarelli, M.G., Finlay, B.B., 1993. Methods to study bacterial invasion. *Journal of Microbiological Methods* 23, 119–125. 227–240.
- Taranto, M.P., Medici, M., Perdigon, G., Holdago, A.P.R., Valdez, G.F., 1998. Evidence for cholesterolemic effect of *Lactobacillus reuteri* in hypercholesterolemic mice. *Journal of Dairy Science* 81, 2336–2340.
- Taranto, M.P., Perez-Martinez, G., de Valdez, G.F., 2006. Effect of bile acid on the cell membrane functionality of lactic acid bacteria for oral administration. *Research in Microbiology* 157, 720-725.
- Taylor, G.R.J., Williams, C.M., 1998. Effects of probiotics and prebiotics on blood lipids. *British Journal of Nutrition* 80, suppl.2, S225-S230.
- Temmerman, R., Pot, B., Huys, G., Swings, J., 2002. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *International Journal of Food Microbiology* 81, 1-10.
- Thompson, W.G., Gick, M., 1996. Irritable bowel syndrome. *Seminars in Gastro-intestinal Diseases* 7 (4), 217–229.
- Toba, T., Virkola, R., Westerlund, B., Björkman, Y., Sillanpää, J., Vartio, T., Kalkkinen, N., Korhonen, T.K., 1995. A collagen-binding S-layer protein in *Lactobacillus cirpatus*. *Applied and Environmental Microbiology* 61, 2467-2471.
- Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.L., Cacarella, C., Lamata, M., Amorenta, B., Leiva, J., Penandés, J.R., Lasa, I., 2001. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Applied and Environmental Microbiology* 67, 4538-4545.



- Tomita, H., Fujimoto, S., Tanimoto, K., Ike, Y., 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pY117. *Journal of Bacteriology* 178, 3585-3593.
- Tuomola, E.M. (née Lehto), Salminen, S.J., 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International Journal of Food Microbiology* 41, 45-51.
- Tuomola, E.M., Ouwehand, A.C., Salminen, S.J., 1999. *FEMS Immunology and Medical Microbiology* 26, (2), 137-142.
- Tyrus, F., 1996. Nutritional care in Intestinal Disease. In: L.K. Mahan, L.K. Escott-Stump, S. (Eds.), *Krause's Food, Nutrition and Diet Therapy*, 9<sup>th</sup> ed. W.B. Saunders Company, U.S.A, pp. 626-627.
- Vallor, A.C., Antonio, M.A.D., Hawse, S.E., Hillier, S.L., 2001. Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. *Journal of Infectious Diseases* 184, 1431-1436.
- Vandenberg, P.A., 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiology Reviews* 12, 221-238.
- Vanderhoof, J.A., Whitney, D.B., Antonsson, D.L., 1999. *Lactobacillus* GG in the prevention of antibiotic-associated diarrhoea in children. *Journal of Pediatrics* 135, 564-568.
- Van Niel, C.W., Feudtner, C., Garrison, M.M., Christakis, D.A., 2002. *Lactobacillus* therapy for acute infectious diarrhoea in children: a meta-analysis. *Pediatrics* 109, 678-684.
- Velraeds, M.C., van der Belt, B., van der Mei, H.C., Reid, G., Busscher, H.J., 1998. Interference in initial adhesion of uropathogenic bacteria and yeasts to silicone rubber by a *Lactobacillus acidophilus* biosurfactant. *Journal of Medical Microbiology* 49, 790-794.
- Ventura, M., Jankovic, I., Walker, D.C., Pridmore, R.D., Zink, R., 2002. Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Applied and Environmental Microbiology* 68, 6172-6181.
- Vesterlund, S., Palta, J., Karp, M., Ouwehand, A.C., 2005. Adhesion of bacteria to resected human colonic tissue: Quantitative analysis of bacterial adhesion and viability. *Research in Microbiology* 156, 238-244.
- Vesterlund, S., Vankerckhoven, V., Saxelin, M., Goossens, H., Salminen, S., Ouwehand, A.C., 2007. Safety assessment of *Lactobacillus* strains: Presence of putative risk factors in faecal, blood and probiotic isolates. *International Journal of Food Microbiology* 116, 325-331.



- Vidal, K.A., Donnet-Hughes, Granato, D., 2002. Lipoteichoic acids from *Lactobacillus johnsonii* strain La1 and *Lactobacillus acidophilus* strain La10 antagonize the responsiveness of human intestinal epithelial HT29 cells to lipopolisaccharide and Gram-negative bacteria. *Infection and Immunity* 70, 2057-2064.
- Vidgrén, G., Palva, I., Pakkanen, R., Lounatmaa, K., Palva, A., 1992. S-layer protein gene of *Lactobacillus brevis*: Cloning by polymerase chain reaction and determination of the nucleotide sequence. *Journal of Bacteriology* 174, 7419–7427.
- Viljanen, M., Savilahti, E., Haahtela, T., Juntunen-Backman, K., Korpela, R., Poussa, T., 2005. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. *Allergy* 60, 494–500.
- Vincent, S., Knight, R.G., Green, M., Sahm, D.F., Shlaes, D.M., 1991. Vancomycin susceptibility and identification of motile enterococci. *Journal of Clinical Microbiology* 29, 2335–2337.
- Wadström, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S., Gullmar, B., 1987. Surface properties of lactobacilli isolated from the small intestine of pigs. *Journal of Applied Bacteriology* 62, 513-520.
- Waters, C.M., Antiporta, M.H., Murray, B.E., Dunny, G.M., 2003. Role of the *E. faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *Journal of Bacteriology* 185, 3613-3623.
- Wang, M.F., Lin, H.C., Wang, Y.Y., Hsu, C.H., 2004. Treatment of perennial allergic rhinitis with lactic acid bacteria. *Pediatric Allergy and Immunology* 15, 152–158.
- Weinstein, D.L., O'Neill, B.L., Hone, D.M., Metcalf, E.S., 1998. Differential early interactions between *Salmonella enterica* serovar *Typhi* and two other pathogenic *Salmonella* serovars with intestinal epithelial cells. *Infection and Immunity* 66, 2310-2318.
- Wells, C.M., Maddaus, M.A., Simmons, R.L., 1988. Proposed mechanisms for the translocation of intestinal bacteria. *Reviews in Infection and Diseases* 10, 958-979.
- WHO, 1997. The Medical Impact of the Use of Antimicrobials in Food Animals. Report of a WHO Meeting, Berlin, Germany, 13–17 October. WHO, Geneva.
- Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC., 1998). The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet* 351, 1225–1232.

- Yang, Z., Suomalainen, T., Mäyrä-Mäkinen, A., Huttenen, E., 1997. Antimicrobial activity of 2-pyrrolidone-5-carboxylic acid produced by lactic acid bacteria. *Journal of Food Protection* 60, 786-790.
- Yazdanbakhsh, M., Kremsner, P.G., van Ree, R., 2002. Allergy, parasites, and the hygiene hypothesis. *Science* 19, 490–494.
- Yokokura, T., 1994. Antitumour and immunostimulating activity of *Lactobacillus casei*. *Japanese Dairy Food Science* 43, A141–A150.
- Young, R.J., Huffman, S., 2003. Probiotic use in children. *Journal of Pediatric Health Care* 17, 6, 277-283.
- Yu, B., Tsen, H.Y., 1993. *Lactobacillus* cells in the rabbit digestive tract and the factors affecting their distribution. *Journal of Applied Bacteriology* 75, 269-275.
- Zárate, G., Pérez, A.C., González, S., Oliver, G., 2000. Viability and  $\beta$ -galactosidase activity of diary propionibacteria subjected to digestion by artificial gastric and intestinal fluids. *Journal of Food Protection* 63, 1214-1221.

# CHAPTER 3

**Evaluation of *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 as probiotics and growth studies in a model simulating an infant gastro-intestinal tract**

Prepared for publication in International Journal of Food Microbiology

## **Evaluation of *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 as probiotics and growth studies in a model simulating an infant gastro-intestinal tract**

**M. Botes, C.A. Van Reenen, L.M.T. Dicks**

*Department of Microbiology, University of Stellenbosch, 7600, Stellenbosch, South Africa*

### **Abstract**

*Enterococcus mundtii* ST4SA produces a broad-spectrum antimicrobial peptide with activity against Gram-positive and Gram-negative bacteria. *Lactobacillus plantarum* 423 produces a bacteriocin active against a number of Gram-positive bacteria. Survival of the two strains was studied in a model simulating an infant gastro-intestinal tract (GIM) and was compared to commercial probiotic strains. Infant milk formulations were used as substrate. Changes in pH were controlled by the addition of 0.5 M HCl or 0.5 M NaOH. Bile salt and pancreatic juice were added to the duodenum vessel. Intestinal flow was controlled by peristaltic pumps receiving signals from a computer program. Strains ST4SA and 423 withstood low pH and elevated bile salts. Both strains are resistant to amoxicillin, cefadroxil, roxithromycin, meloxicam, doxycycline, novobiocin, and ibuprofen, and painkillers codeine terprim hydrate, aminobenzoic acid, metamizole, aspirin and paracetamol. Strain 423 is sensitive to vancomycin and does not contain genes encoding gelatinase, cell aggregation, enterococcus surface protein, hemolysin, non-cytolysin  $\beta$ -hemolysin and enterococcus endocarditis antigen. Genes encoding cytolysin, non-cytolysin  $\beta$ -hemolysin III and cell aggregation were detected on the genome of strain ST4SA, but they were not expressed. The reason for the latter is unknown. Both strains inhibited the growth of *Listeria monocytogenes* ScottA in the GIM. Survival of the strains improved when used in combination and compared well with the survival of commercially available probiotics.

*Keywords:* *Enterococcus mundtii* ST4SA, *Lactobacillus plantarum* 423, Probiotics, GIM

## 1. Introduction

A number of lactic acid bacteria have been classified as probiotics and are either incorporated into functional foods or marketed as lyophilized cells in capsules (Fuller, 1989; Svensson, 1999). Health benefits described for probiotics include prevention or treatment of infectious diseases, irritable bowel syndrome, allergies, lactose intolerance, colon cancer and reduction of serum cholesterol levels (Andersson et al., 2001).

Criteria for selection of a probiotic vary, but usually includes the ability to adhere to mucus and epithelial cells (Gorbach, 2002), and survival at low pH (1.0 to 3.0) and bile salts of approximately 0.3 % (Mainville et al., 2005). Recent reports of lactic acid bacteria, especially *E. faecalis* and *E. faecium*, associated with nosocomial infections (Jones et al., 1997; Cannon et al., 2005; Salminen et al., 1998) have alerted scientists to screen potential probiotic strains for virulence factors (Edwards, 2000; Mundy et al., 2000; Routsis et al., 2000).

Most probiotic tests were performed *in vitro* (Alander et al., 1997; Isolauri et al., 1994; Matsumura et al., 1999; Minekus et al., 1995; Sarem-Damerdjil et al., 1995). Models have been developed to simulate gastro-intestinal conditions. The simulated human intestinal microbial ecosystem (SHIME), developed by Molly and co-workers (1993) was a five-stage reactor with a two-step “fill and draw” system that represented the small intestine and a three-step reactor representing the large intestine. The medium in each vessel was kept in suspension by a magnetic stirrer and the pH controlled by probes linked to control units. Minekus and co-workers (1995) developed an upper gastro-intestinal model with four compartments, representing the stomach, duodenum, jejunum and ileum. Each compartment consisted of a glass exterior with flexible inner silicon tubing, connected by peristaltic valves which regulated the flow of nutrients to each compartment. Macfarlane and co-workers (1998) developed a three-stage compound continuous culture system simulating the proximal colon. The pH was automatically controlled and each vessel was flushed with CO<sub>2</sub>. The most recent gastro-intestinal model developed by Mainville et al. (2005) simulated the stomach and duodenum. Each vessel was equipped with pH and temperature probes and entry ports for medium, HCl, NaOH and ox-bile. The medium in each reactor was kept in suspension by a magnetic stirrer and the temperature maintained at 37°C with a circulating water bath.

In this study, *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 were evaluated as probiotics by studying their growth in infant milk formulations, inhibition of *Listeria monocytogenes*, growth at low pH and in the presence of elevated bile salts, susceptibility to antibiotics, anti-inflammatory drugs and painkillers, and presence of virulence factors. Survival of the strains in the gastro-intestinal tract (GIT) was studied by using infant milk formulations as substrate in a computerized gastro-intestinal model (GIM) designed to simulate nutrient flow through the GIT of an infant.

## 2. Materials and Methods

### 2.1 Bacterial strains and growth conditions

*Enterococcus mundtii* ST4SA, isolated from soybeans (Knoetze, 2006), and *Lactobacillus plantarum* 423 isolated from sorghum beer (Van Reenen et al. 1998), were cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, SA) for 18 h at 37°C. *Lactobacillus rhamnosus* R-11 and *Lactobacillus acidophilus* La5, isolated from functional dairy products, *Lactobacillus reuteri* isolated from a probiotic supplement, and *Lactobacillus johnsonii* La1, *Lactobacillus rhamnosus* GG and *Lactobacillus casei* Shirota (received from W.H. Holzapfel, Institute of Hygiene and Toxicology, Karlsruhe, Germany) were cultured in MRS broth (Biolab) at 37°C for 18 h. *Listeria monocytogenes* ScottA, cultured in Brain Heart Infusion (BHI) (Biolab) served as a model of a pathogen and as target strain for determination of antimicrobial activity.

### 2.2 Growth of *E. mundtii* ST4SA and *L. plantarum* 423 in infant milk formulations

*E. mundtii* ST4SA and *L. plantarum* 423 were inoculated, separately, into 10 ml MRS broth and incubated at 37°C for 18 h. The cultures were washed (8000 x g, 15 min, 18°C) with sterile saline (0.75 %, w/v, NaCl) and the pellets resuspended into 200 ml N1 (57.8 % maltodextrin, 6 % demineralised whey, 6 % skimmed milk, 25.9 % vegetable fat, vitamins and minerals) and 200 ml L1 2 (54.7 % maltodextrin, 1.7 % fructo-oligosaccharides, 18.3 % skimmed milk, 18.2 % milk fat, vitamins and minerals), respectively. Incubation was at 37°C. Samples were taken every two hours for 21 h and plated onto MRS agar (Biolab). Antimicrobial activity was determined using the agar spot method, as described by Uhlman et al. (1992). Aliquots of 10 µl cell-free culture supernatant fluid were spotted onto an agar plate (0.7 % w/v agar) seeded with active growing cells of *L. monocytogenes* ScottA (approximately  $10^6$  cells ml<sup>-1</sup>). Plates were incubated at 37°C for 18 h. A clear zone of inhibition of at least 2 mm in diameter was recorded as positive.

### 2.3 Design of the infant gastro-intestinal model

The gastro-intestinal model (GIM), shown in Fig. 1, was developed to simulate nutrient flow through an infant gastro-intestinal tract, aged between 6 and 12 months, and was a combination of the models described by Molly et al. (1993) and Mainville et al. (2005). The GIM consists of four vessels, representing the stomach, duodenum, jejunum, and ileum. Pancreatic juice and bile salts [2.4 g NaHCO<sub>3</sub>, 0.18 g pancreatin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 1.2 g Ox-bile (Oxoid Ltd, Hampshire, England)] were kept in a separate vessel (Fig. 1). The reservoir vessel contained 400 ml sterile saliva buffer (1.24 g NaCl, 0.44 g KCl, 0.04 g CaCl<sub>2</sub>, 0.24 g NaHCO<sub>3</sub>). Vessels were connected with autoclavable Nalgene tubing (Fig. 1). Peristaltic pumps were linked to a LabPro Interface system (Vernier Software & Technology, Beaverton, USA), controlled by a computer program designed by J. Kistner and R. Kistner (Information Technology, University of Stellenbosch). All components, except the computer, were housed in a 37°C room.

The substrate was pumped from the reservoir to the stomach, and then to the duodenum, jejunum and ileum at specific time intervals (Table 1). The pancreatic juice and bile salts (200 ml) were injected into the duodenum after 2.5 h. The flow speed of the peristaltic pumps was controlled at 104 ml min<sup>-1</sup> (Van den Driessche et al., 1999). The pH in the stomach was gradually decreased to 3.7 with the addition of 0.5 M HCl to simulate milk digestion in infants. The pH in the ileum vessels was kept at 6.0 with the addition of 0.5 M HCl and the pH in the duodenum and jejunum vessels kept at 6.5 with the addition of 0.5 M NaOH. Changes in pH were recorded with pH probes (Vernier Software & Technology, Beaverton, OR), linked to peristaltic pumps (Fig. 1).

### 2.4 Preparation of starter cultures and operation of the GIM

*E. mundtii* ST4SA and *L. plantarum* 423 were inoculated, separately, into 10 ml MRS broth (Biolab) and incubated at 37°C for 18 h. The cultures were then transferred to 200 ml MRS broth (Biolab) and incubated at 37°C to OD<sub>600</sub> = 1.5. The cells were harvested (8000 x g, 15 min, 18°C), washed with half-strength saliva buffer (Marteau et al., 1997) and the pellets resuspended in 10 ml of the same buffer. These cell suspensions were used to inoculate the reservoir (400 ml saliva buffer), supplemented with either 58.2 g N1 or 65.3 g L1 2. Final cell numbers in the reservoir were 1.0 x 10<sup>8</sup> cfu/ml and the pH adjusted to approximately 6.8 with sterile 0.5 M NaOH. In a separate experiment, the reservoir was inoculated with a combination (1:1) of strains ST4SA and 423 (total = 1 x 10<sup>8</sup> cfu/ml). Conditions were the same.

Samples were collected with sterile tubing (1 mm diameter) connected to a 1 ml sterile syringe. Viable cell numbers of strains ST4SA and 423 were determined by plating onto Enterococcus Specific Agar (Difco, Becton, Dickinson and Company, Le Pont de Claix, France) and MRS agar (Biolab), respectively. All plates were incubated at 37 °C for 48 h. Antimicrobial activity was determined using the agar spot method, as described before.

The experiment was repeated by using *L. rhamnosus* R-11, *L. acidophilus* La5, *L. reuteri*, *L. johnsonii* La1, *L. rhamnosus* GG and *L. casei* Shirota as probiotics. Conditions were the same, except that only N1 was used as substrate. Viable cell numbers were determined by plating onto MRS agar (Biolab) and antimicrobial activity determined as before. Each experiment was conducted in triplicate.

In a separate experiment, the GIM colonized with ST4SA, 423 or a combination of the two strains ( $1.0 \times 10^8$  cfu/ml), was contaminated with *L. monocytogenes* ScottA ( $1.0 \times 10^4$  cfu/ml). The experiment was conducted as described before. Cell counts of *L. monocytogenes* ScottA were determined by plating onto Listeria agar (Merck). The control was *L. monocytogenes* ScottA grown in the absence of *E. mundtii* ST4SA and *L. plantarum* 423.

### 2.5 Screening for bile-salt hydrolase (BSH) activity

*E. mundtii* ST4SA, *L. plantarum* 423, *Lactobacillus rhamnosus* R-11, *L. acidophilus* La5, *L. reuteri*, *L. casei* Shirota, *L. rhamnosus* GG and *L. johnsonii* La1 were screened for BSH activity by spotting 10 µl of cultures ( $OD_{600} = 1.5$ ) onto MRS agar (Biolab), supplemented with 0.5% (w/v) taurodeoxycholic acid (Sigma) and  $0.37 \text{ g L}^{-1}$   $\text{CaCl}_2$ . Plates were incubated for 72 h at 37°C in an anaerobic jar with a gas generating kit (Oxoid). Formation of a precipitation zone surrounding the colonies was regarded BSH positive.

### 2.6 Susceptibility to antibiotics, anti-inflammatory drugs and painkillers

Strains ST4SA and 423 were tested for susceptibility to ampicillin, bacitracin, cephazolin, chloramphenicol, ciprofloxacin, compound sulphonamides, cloxacillin, erythromycin, metronidazole, methicillin, neomycin, novobiocin, nystatin, ofloxacin and oxacillin (Oxoid) by using the disc diffusion method (Charteris et al., 1998). The susceptibility of *E. mundtii* ST4SA, *L. plantarum* 423, *L. casei* Shirota, *L. rhamnosus* GG and *L. johnsonii* La1 to commercially available antibiotics, anti-inflammatory medicaments and painkillers was also determined (see



Table 2). Strains were inoculated, separately, into 10 ml MRS broth (Biolab) and incubated at 37°C for 18 h and imbedded into MRS soft agar (1.0 %, w/v) (Biolab) at 10<sup>6</sup> cfu/ml. Ten µl of each medicament was spotted onto the surface of the agar and the plates incubated for 18 h.

### 2.7 Screening for the presence of virulence factors

*E. mundtii* ST4SA, *L. plantarum* 423, *L. casei* Shirota, *L. johnsonii* La1, *L. rhamnosus* GG, *E. faecium* T8 and *E. faecium* ST311LD were cultured in MRS broth (Biolab) at 37°C to OD<sub>600</sub> = 1.5. DNA was isolated according to Archimbaud et al. (2002). Plasmid DNA of *E. mundtii* ST4SA was isolated by using the Qiagen plasmid midi kit (Qiagen, Inc., Valencia, USA). Primers designed from genes encoding virulence (Table 3) were used to amplify the genomic and plasmid DNA. Amplified DNA fragments of expected sizes were isolated from the gels (Omar et al., 2004), sequenced on an ABI Genetic Analyzer 3130XL Sequencer (Applied Biosystem, South Africa, Pty, Ltd.) by using BigDye Terminator Cycle Chemistry (Biosystems, Warrington, UK), and searched for homologous sequences in GenBank using BLAST ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

Production of gelatinase was determined by streaking single colonies of each strain onto MRS agar, supplemented with 3 % (w/v) gelatin (BDH Laboratory Supplies, Poole, England). Plates were incubated at 37 °C for 24 h followed by incubation at 4 °C for 5 h. Colonies with surrounding opaque zones were regarded gelatinase positive (Eaton & Gasson, 2002).

Production of an aggregation substance was determined as described by Franz et al. (2001). *Enterococcus faecalis* OGIX, producer of a pheromone, was cultured in Todd Hewitt broth (Difco, Becton Dickinson, Le Pont de Claix, France) at 37 °C for 18 h. Cell-free culture supernatant (200 µl) of *E. faecalis* OGIX was added (0.5 % v/v) to each of the test strains and microscopically examined for cell clumping after 2, 4, 8 and 24 h.

Production of haemolysin was determined by streaking the strains onto Columbia Agar (Merck, Merck Chemicals, Gauteng, SA), supplemented with 5% (v/v) sheep blood. Plates were incubated for 72 h at 37 °C in anaerobic jars with gas generating kits (Oxoid). The presence of zones surrounding the colonies indicated β-haemolysis. The absence of zones was interpreted as no haemolysis (Semedo et al., 2003).

### 3. Results

#### 3.1 Growth of *E. mundtii* ST4SA and *L. plantarum* 423 in infant milk formulations

*E. mundtii* ST4SA reached  $3.5 \times 10^9$  cfu/ml after 12 h in N1 and 18 h in L1 2. Highest activity of peptide ST4SA (25 600 AU/ml) was recorded between 8 and 15 h in N1 and between 8 and 21 h in L1 2, followed by a decrease to 12 800 AU/ml after 18 h in N1. The culture pH decreased from 6.8 to 4.2 in N1 and from 6.8 to 4.4 in L1 2 after 21 h of growth (Fig. 2). *L. plantarum* 423 reached highest cell numbers ( $4.0 \times 10^{10}$  cfu/ml) after 12 h in N1 and 15 h in L1 2 ( $1.4 \times 10^{10}$  cfu/ml). Plantaricin 423 activity of 1600 AU/ml was recorded after 12 h in N1. Highest activity (6400 AU/ml) was recorded between 10 and 18 h in L1 2, followed by a decrease to 3200 AU/ml in the same substrate. The pH decreased from 6.8 to 4.3 after 21 h in N1 and from pH 6.8 to 5.6 in L1 2 (Fig. 3).

#### 3.2 Growth and survival of *E. mundtii* ST4SA and *L. plantarum* 423 in the model

Cell numbers of *E. mundtii* ST4SA decreased from  $1.0 \times 10^8$  cfu/ml to  $7.5 \times 10^6$  cfu/ml after 2.5 h in the stomach vessel filled with N1 and to  $8.5 \times 10^7$  cfu/ml over the same period in the presence of L1 2 (Fig. 4). Addition of bile and pancreatin to the duodenum and jejunum led to a further decrease in cell numbers of *E. mundtii* ST4SA after 4 h ( $1.6 \times 10^6$  in N1 and  $9.9 \times 10^6$  in L1 2). Cell numbers increased by one log in the ileum (to  $3.1 \times 10^7$  cfu/ml in N1 and  $8.4 \times 10^7$  in L1 2). Peptide ST4SA activity decreased from 25 600 AU/ml to 12 800 AU/ml in the stomach, duodenum and jejunum, but increased to 25 600 AU/ml in the ileum in both substrates.

*L. plantarum* 423 decreased from  $1.0 \times 10^8$  cfu/ml to  $2.2 \times 10^7$  cfu/ml in the stomach vessel filled with N1 and to  $1.0 \times 10^7$  cfu/ml when filled with L1 2. Bile and pancreatin stimulated the growth of *L. plantarum* 423 (from  $2.2 \times 10^7$  to  $3.6 \times 10^7$  cfu/ml in N1 and from  $1.0 \times 10^7$  to  $8.0 \times 10^8$  cfu/ml in L1). In N1, cell numbers increased to  $1.5 \times 10^8$  cfu/ml in the jejunum and decreased to  $2.4 \times 10^7$  cfu/ml in the ileum. In L1 2, cell numbers decreased to  $5.4 \times 10^8$  cfu/ml in the jejunum and to  $2.8 \times 10^8$  cfu/ml in the ileum. Plantaricin 423 activity decreased from 800 AU/ml to 400 AU/ml in the stomach and duodenum with N1 as substrate. Activity increased to 800 AU/ml in the jejunum and decreased to 400 AU/ml in the ileum. With L1 2 as substrate, the activity of bacteriocin 423 remained at 800 AU/ml in the stomach, duodenum and jejunum, but increased to 1600 AU/ml in the ileum.

Less fluctuation in cell numbers was observed when *E. mundtii* ST4SA and *L. plantarum* 423 were used in combination, irrespective of the substrate. *E. mundtii* ST4SA increased from  $1.0 \times 10^8$  cfu/ml to  $2.0 \times 10^8$  cfu/ml after 8.5 h in the presence of *L. plantarum* 423. *L. plantarum* 423 increased from  $1.0 \times 10^8$  cfu/ml to  $3.6 \times 10^8$  cfu/ml and to  $4.8 \times 10^8$  cfu/ml in the presence of *E. mundtii* ST4SA over the same period (Fig. 4).

*L. rhamnosus* GG produced the highest cell numbers in the stomach ( $5.0 \times 10^8$  cfu/ml), the duodenum ( $9.0 \times 10^8$  cfu/ml) and jejunum ( $5.0 \times 10^8$  cfu/ml) after 2.5, 4.5 and 6.5 h, respectively. Growth of *L. reuteri* was slower and reached  $1.3 \times 10^8$  cfu/ml in the ileum after 8.5 h. Cell numbers of *L. johnsonii* La1 decreased from  $1.0 \times 10^8$  cfu/ml to  $3.0 \times 10^7$  cfu/ml in the stomach and increased to  $7.0 \times 10^8$  cfu/ml after 6 h in the duodenum, jejunum and ileum. *L. casei* Shirota decreased from  $1.0 \times 10^8$  cfu/ml to  $1.5 \times 10^7$  cfu/ml in the stomach, but increased to  $1.0 \times 10^8$  cfu/ml after a further 6 h in the duodenum, jejunum and ileum. *L. acidophilus* La5 and *L. rhamnosus* R-11 decreased by two log cycles in the presence of acid and bile (Fig. 5).

*L. monocytogenes* ScottA remained at  $1.0 \times 10^4$  cfu/ml in the stomach, but increased to  $1.0 \times 10^6$  cfu/ml in the duodenum. Growth of ScottA was repressed by *E. mundtii* ST4SA and cell numbers remained at  $1.0 \times 10^4$  cfu/ml. In the presence of *L. plantarum* 423, cell numbers of ScottA decreased from  $1.0 \times 10^4$  cfu/ml to  $2.5 \times 10^3$  cfu/ml in the duodenum and jejunum, but increased to  $8.0 \times 10^3$  cfu/ml in the ileum (results not shown).

### 3.3 Screening of bile-salt hydrolase (BSH) activity

No bile-salt hydrolase activity was detected in any of the strains.

### 3.4 Susceptibility to antibiotics, anti-inflammatory drugs and painkillers

Growth of *E. mundtii* ST4SA was inhibited by ampicillin, bacitracin, cephalazolin, chloramphenicol, ciprofloxacin, erythromycin, novobiocin, ofloxacin, oxacillin, rifampicin and tetracycline (Table 2). Growth of *L. plantarum* 423 was inhibited by ampicillin, bacitracin, cephalazolin, chloramphenicol, compound sulphonamides, erythromycin, novobiocin, ofloxacin, oxacillin, rifampicin and tetracycline (Table 2). *E. mundtii* ST4SA and *L. plantarum* 423 were resistant to commercially available  $\beta$ -lactam penicillins (Promoxil and Cipadur) and macrolides (Roxibidd and Doximal). Similar results were recorded for *L. casei* Shirota, *L. rhamnosus* GG and *L. johnsonii* La1. *L. plantarum* 423 was the only strain resistant to Ciprofloxacin.

### 3.5 Screening for the presence of virulence factors

*E. mundtii* ST4SA contained the genes encoding cytolysin ( $\beta$ -hemolysin), non-cytolysin (beta hemolysin III), and cell aggregation (Table 3). No virulence genes were recorded for *L. plantarum* 423, *L. casei* Shirota, *L. rhamnosus* GG and *L. johnsonii* La1. Aggregation substances (AS) and gelatinase were not produced and no haemolytic activity was detected in any of these strains. *E. faecium* T8 and ST311LD contained the *E. faecium* endocarditis antigen, *E. faecalis* endocarditis antigen, cytolysin ( $\beta$ -hemolysin) and non-cytolysin (beta hemolysin III). None of the strains contained genes encoding vancomycin resistance, gelatinase, adhesin to collagen or the enterococcus surface protein.

## 4. Discussion

Infant milk formulations N1 and L1 2 provided sufficient growth factor requirements for *E. mundtii* ST4SA and *L. plantarum* 423. However, both strains preferred L1 2 (maltodextrin, fructo-oligosaccharides and skimmed milk) as substrate. This corresponded to results obtained for strain 423 in MRS broth supplemented with 1 % (w/v) inulin and 1 % (w/v) fructo-oligosaccharides (Brink et al., 2006). Medium supplemented with these oligosaccharides also supported growth of strain 423 at low pH and at high bile concentrations. Similar results were recorded for *Lactobacillus curvatus* DF38, *Lactobacillus salivarius* 241, *L. casei* LHS and *Pediococcus pentosaceus* 34 grown in the presence of 1 % inulin and 1 % (w/v) fructo-oligosaccharides (Brink et al., 2006). This phenomenon has also been reported for other lactic acid bacteria (Kontula et al., 1998). The high activity levels recorded for peptide ST4SA and plantaricin 423 in L1 2 may be due to the metabolism of fructo-oligosaccharides that may stimulate the production of bacteriocins.

Sensitivity of *E. mundtii* ST4SA to low pH, elevated bile levels and pancreatin suggests that only a few cells will survive conditions in the stomach and duodenum, but that the cells will recover in the ileum. Acidic conditions repressed the growth of *L. plantarum* 423 and it is safe to assume that these bacteria will not survive the conditions in the stomach. Growth will, however, commence in the duodenum, irrespective of the presence of bile and pancreatin. High cell numbers recorded for both strains in the ileum suggests that they may survive conditions in the colon. Increased survival recorded when strains ST4SA and 423 were used in combination suggests symbiotic growth, possibly the fermentation of complex sugars to fermentable

carbohydrates. It is well known that multispecies probiotics create their own niche to improve survival under conditions of stress (Timmerman et al., 2004).

Food-borne pathogens tolerate 500 to 1000 ml bile secreted from the gallbladder (Hofmann, 1994). Growth of *L. monocytogenes* ScottA was not inhibited by pH 3.7 and 0.6 % (w/v) bile in the GIM. This corresponds to previous studies that showed *L. monocytogenes* is not affected by 0.3 % to 30 % (v/v) bile from human, bovine or porcine (De Boever & Verstraete, 1999; Hofmann et al., 1983). Genes encoding for capsule formation (*capA*), transcriptional regulation (*ZurR*), isoprenoid biosynthesis (*lytB*), a membrane protein (*YxiO*) and a amino acid transporter with a putative role in pH homeostasis (*gadE*) have been described for *L. monocytogenes* LO28, which explains the high tolerance to bile (Begley et al., 2002).

Growth inhibition of *L. monocytogenes* ScottA is ascribed to the production of antimicrobial peptide ST4SA and plantaricin 423. The mode of action and structure of the peptides have been described in previous papers (de Kwaadsteniet et al., 2005; Granger et al., 2005; Todorov et al., 2005; Van Reenen et al., 1998). Both peptides act by dissipating the proton motive force of the target cell.

Growth of *E. mundtii* ST4SA and *L. plantarum* 423 in the GIM superseded that recorded for *L. acidophilus* La5 and *L. rhamnosus* R-11, suggesting that they will colonize the GIT at higher levels. Mainville et al. (2004) has shown that only 0.1 % of cells of *L. rhamnosus* GG survived in a stomach reactor, whereas *L. johnsonii* La1 showed 76 % survival. However, previous studies showed that high cell numbers of *L. rhamnosus* GG reached the colon *in vivo* (Goldin et al., 1992; Mainville et al., 2004; Siitonen et al., 1990). Furthermore, in human trial studies *L. casei* Shirota and *L. johnsonii* La1 survived conditions in the GIT the best (Holzapfel et al., 2001; Mainville et al., 2004; Salminen et al., 1998; Spanhaak et al., 1998). From these results, it is clear that *in vitro* studies have to be substantiated with *in vivo* studies.

Possible mechanisms contributing to the acid and bile resistance of the strains include proton pumps, amino acid decarboxylation and transport (putative role in pH homogenesis), electrogenic transport systems, chaperones involved in repair/degradation of damaged proteins, incremental expression of regulators promoting local or global responses, elements involved in the maintenance of the cell envelope, energy metabolism, and fatty acid or isoprenoid biosynthesis

(Begley et al., 2002; Breton et al., 2002; Cotter & Hill, 2003). The cell could be protected from harsh conditions by alterations in the cell wall (Bron, Hoffer et al., 2004).

The correlation of BSH activity and high tolerance to bile salts is debatable (Pinto et al., 2006). *Lactobacillus* spp. may use deconjugation of bile salts as a detoxification mechanism (De Smet et al., 1995; Tannock et al., 1989). Superior toxicity of the deconjugated salts may also affect the viability of cells (Grill et al., 2000). Resistance of *E. mundtii* ST4SA and *L. plantarum* 423 to ox-bile cannot be correlated with BSH activity and confirms recent studies on bile-salt hydrolase (Moser & Savage, 2001; Schmidt et al., 2001).

Enterococci are resistant to a broad range of antibiotics (Landman & Quale, 1997; Leclercq, 1997; Murray, 1990). *E. mundtii* ST4SA showed resistance to beta-lactam penicillins, macrolides, tetracyclines, quinolones and anti-inflammatory medicaments. No inhibition by norfloxacin, an antibiotic for urinary tract infections, the anti-inflammatory medicaments, diclofenak, Na<sup>+</sup>-hydrogen carbonate, benzoic acid and painkillers, metamizole codeine terprim hydrate amino benzoic acid and paracetamol was detected. The antibiotic susceptibility profile of *L. plantarum* 423 correlates with that reported for other lactobacilli and is similar to *E. mundtii* ST4SA. *L. plantarum* 423 is the only strain resistant to the quinolone ciprofloxacin. Some lactobacilli have a high natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethoprim/sulphamethoxazole, and vancomycin (Danielsen & Wind, 2003). However, the levels of susceptibility of *Lactobacillus* spp. to various antimicrobial agents are species-dependant (Danielsen & Wind, 2003). The antibiotic resistance profile of 55 European probiotic strains revealed resistance to kanamycin (79 % of the isolates), vancomycin (65 %), tetracycline (26 %), penicillin G (23 %), erythromycin (16 %) and chloramphenicol (11 %). Overall 68.4 % of the isolates showed resistance against multiple antibiotics, including intrinsic resistance (Temmerman et al., 2002). Growth of probiotic bacteria is either inhibited by the majority of antibiotics or they are multi-resistant. In the case of multi-resistance the probiotic can be co-administrated with antibiotics. The possible negative effects are resistance transfer directly or indirectly via the commensal flora from probiotic strains to pathogenic bacteria. Probiotics could acquire resistance genes from human commensals and when these probiotics cause infection mainly in immunocompromised patients, only a limited number of antibiotics could be used to treat the patient (Courvalin, 2006).

The virulence of lactic acid bacteria involves more than multi-drug antibiotic resistance (Franz and Holzapfel, 2004). Virulence traits determined in clinical isolates may be associated with one or more stages of infection (Franz et al., 2003; Jett et al., 1994). Three virulence factors were found in *E. mundtii* ST4SA, although no functional homology of the genes was detected which indicate low virulence potential. The AS found in the plasmid of *E. mundtii* ST4SA is encoded on pheromone-responsive plasmids. These pheromone-responsive plasmids may contribute to pathogenesis of infection by enterococci through different mechanisms (Foulquié Moreno et al., 2006; Franz et al., 2003). No clumping response was detected in *E. mundtii* ST4SA and the AS gene is therefore not expressed. No virulence factors were found in *L. plantarum* 423. The safety of both strains was confirmed in rat studies (unpublished data).

The GIM proved useful to predict the survival of *E. mundtii* ST4SA and *L. plantarum* 423 in the GIT of infants and provided an indication as to the area where each strain would colonize. Both strains inhibited the growth of *L. monocytogenes* ScottA in the GIM. These characteristics, and the absence of virulence factors, render *E. mundtii* ST4SA and *L. plantarum* 423 good candidates as probiotics. Future modifications of the GIM may include the incorporation of more enzymes involved in food digestion and the use of different food matrixes.

### Acknowledgements

Noelle Basson for technical assistance and the National Research Foundation (NRF) of South Africa for funding.

### References

- Alander, M., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., von Wright, A., 1997. Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. *Letters of Applied Microbiology* 24, 361–364.
- Andersson, H., Asp, N.-G., Bruce, A., Roos, A., Wadström, T., Wold, A.E., 2001. Health effects of probiotics and prebiotics. A literature review on human studies. *Scandinavian Journal of Nutrition* 45, 58-75.
- Archimbaud, C., Shankar, N., Forestier, C., Baghdayan, A., Gilmore, M.S., Charbonné, F. Jolya, B., 2002. *In vitro* adhesive properties and virulence factors of *Enterococcus faecalis* strains. *Research in Microbiology* 153, 75–80.



- Begley, M., Gahan, C.G., Hill, C., 2002. Bile stress response in *Listeria monocytogenes* LO28: Adaptation, cross-protection, and identification of genetic loci involved in bile resistance. *Applied and Environmental Microbiology* 68, 6005-6012.
- Breton, Y.L., Maze, A., Hartke, A., Lemarinier, S., Auffray, Y., Rince, A., 2002. Isolation and characterization of bile salts-sensitive mutants of *Enterococcus faecalis*. *Current opinions in Microbiology* 45, 434-439.
- Brink, M., Todorov, S.D., Martin, J.H., Senekal, M., Dicks, L.M.T., 2006. The effect of prebiotics on production of antimicrobial compounds, resistance to growth at low pH and in the presence of bile, and adhesion of probiotic cells to intestinal mucus. *Journal of Applied Microbiology* 100, 813-820.
- Bron, P.A., Hoffer, S.M., Van Swam, I.I., de Vos, W.M., Kleerebezem, M., 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. *Applied Environmental Microbiology* 70 , 310-317.
- Cannon, J. P., Lee, T.A., Bolanos, J.T., Danziger, L.H., 2005. Pathogenic relevance of *Lactobacillus*: a retrospective review of over 200 cases. *European Journal of Clinical Microbiology* 24, 31-40.
- Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K., 1998b. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. *Journal of Food Protection* 61, 1636–1643.
- Cotter, P.D., Hill, C., 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. *Molecular Microbiology* 33, 1152-1161.
- Courvalin, P., 2006. Antibiotic resistance: The pros and cons of probiotics. *Digestive and Liver Disease* 38 (Suppl.2), S261-265.
- Clark, N., Teixeira, L., Facklam, R., Tenover, F., 1998. Detection and differentiation of *vanC1*, *vanC2* and *vanC3* glycopeptide resistance genes in enterococci. *Journal of Clinical Microbiology* 36, 2294–2297.
- Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology* 82, 1 –11.
- De Boever, P., Verstraete, W., 1999. Bile salt deconjugation by *Lactobacillus plantarum* 80 and its implication for bacterial toxicity. *Journal of Applied Microbiology* 87, 345–352.
- De Kwaadsteniet, M., Todorov, S.D., Knoetze, H., Dicks, L.M.T., 2005. Characterization of a 3944 kDa bacteriocin produced by *Enterococcus mundtii* ST15, with activity against



- Gram-positive and Gram-negative bacteria. *International Journal of Food Microbiology* 105, 433-444.
- De Man, J.D., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology* 23, 130-135.
- De Smet, I., Van Hoorde, L., De Saeyer, N., Van de Woestyne, M., Vestraete, W., 1994. *In vitro* study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of lowering through enhanced BSH activity. *Microbial Ecology in Health and Disease* 7, 315-329.
- Eaton, T.J., Gasson, M.J., 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology* 67, 1628-1635.
- Edwards, D.D., 2000. Enterococci attract attention of concerned microbiologists. *American Society for Microbiology News* 66, 540-545.
- Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. *International Journal of Food Microbiology* 106, 1-24.
- Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H., Holzapfel, W.H., 2003. Enterococci in foods-a conundrum for food safety. *International Journal of Food Microbiology* 88, 105-122.
- Franz, C.M.A.P., Holzapfel, W.H., 2004. The genus *Enterococcus*: Biotechnological and safety issues. In: Salminen, S., von Wright, A., Ouwehand, A. (Eds.), *Lactic acid bacteria Microbiological and Functional aspects*, 3<sup>rd</sup> ed. Revised and expanded, Marcel Dekker, inc., pp. 199-247.
- Fuller, R., 1989. Probiotics in man and animals. *Journal of Applied Bacteriology* 66, 365-378.
- Granger, M., Todorov, S., Matthew, M., Dicks, L.M.T., 2005. Growth of *Enterococcus mundtii* ST15 in medium filtrate and purification of bacteriocin ST15 by cation-exchange chromatography. *Journal of Basic Microbiology* 6, 415-425.
- Grill, J.P., Perrin, S., Scheneider, F., 2000. Bile salt toxicity to some bifidobacteria strains: role of conjugated bile salt hydrolase and pH. *Canadian Journal of Microbiology* 46, 878-884.
- Goldin, B.R., Gorbach, S.L., Saxelin, M., Barakat, S., Gualtieri, L., Salminen, S., 1992. Survival of *Lactobacillus* species (strain GG) in human gastro-intestinal tract. *Digestive Diseases and Sciences* 37 (1), 121-128.
- Gorbach, S.L., 2002. Probiotics in the third millennium. *Digestive and Liver Disease* 34 (Suppl.2), 52-57.

- Hechard, Y., Sahl, H.G., 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie* 84, 545-557.
- Hofmann, A. F., Molino, G., Milanese, M., Belforte, G., 1983. Description and stimulation of a physiological pharmacokinetic model for the metabolism and enterohepatic circulation of bile acids in man. *Journal of Clinical Investigations* 71, 1003–1022.
- Holzapfel, W.H., Haberer, P, Geisen, R., Bjötkroth, J., Shillinger, U., 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. *American Journal of Clinical Nutrition* 73, 365S-373S.
- Isolauri, E., Kaila, M., Mykkänen, H., Ling, W.H., Salminen, S., 1994. Oral bacteriotherapy for viral gastroenteritis. *Digestive and Disease Sciences* 39, 2595-2600.
- Jett, B.D., Huycke, M.M., Gilmore, M.S., 1994. Virulence of enterococci. *Clinical Microbiology Reviews* 7, 462-478.
- Jones, R.N., Marshall, S.A., Pfaller, M.A., Wilke, W.W., Hollis, R.J., Erwin, M.E., Edmond, M.B., Wenzel, R.P., the SCOPE Hospital Study Group, 1997. Nosocomial enterococcal blood stream infections in the SCOPE program: antimicrobial resistance, species occurrence, molecular testing results, and laboratory testing accuracy. *Diagnostic Microbiology Infectious Disease* 29, 95–102.
- Knoetze, H., 2006. Characterization of a broad-spectrum anti-microbial peptide from *Enterococcus mundtii* active against bacteria associated with middle-ear infections. MSc. Thesis, Stellenbosch University.
- Landman, D., Quale, J.M., 1997. Management of infections due to resistant enterococci: a review of therapeutic options. *Journal of Antimicrobial Chemotherapy* 40, 161– 170.
- Leclercq, R., 1997. Enterococci acquire new kinds of resistance. *Clinical Infectious Diseases* 24 (Suppl.1), S80– S84.
- Lemcke, R., Bülte, M., 2000. Occurrence of the vancomycin-resistant genes *vanA*, *vanB*, *vanC1*, *vanC2* and *vanC3* in *Enterococcus* strains isolated from poultry and pork. *International Journal of Food Microbiology* 60, 185-194.
- Macfarlane, G.T., Marfarlane, S., Gibson, G.R., 1998. Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial Ecology* 35, 180-187.
- Mainville, I., Arcand, Y., Farnworth, E.R., 2005. A dynamic model that simulates the human upper gastro-intestinal tract for the study of probiotics. *International Journal of Food Microbiology* 99, 287-296.

- Marteau, P., Minekus, M., Havenaar, R., Huis in't Veld, J.H.J., 1997. Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: Validation and the effects of bile. *Journal of Dairy Science* 80, 1031-1037.
- Matsumura, A.T., Saito, T., Arakuni, M., Kitazawa, H., Kawai, Y., Itoh, T., 1999. New binding assay and preparative trial of cell-surface lectin from *Lactobacillus acidophilus* group lactic acid bacteria. *Journal of Dairy Science* 82, 2525-2529.
- Minekus, M., Marteau, P., Havenaar, R., Huis in't Veld, J.H.J., 1995. A multi-compartmental dynamic computer-controlled model simulating the stomach and the small intestine. *Alternatives to Laboratory Animals* 23, 197-209.
- Molly, K., Van de Woestyne, M., Verstraete, W., 1993. Development of a 5-step multi-chamber reator as a simulation of the human intestinal microbial ecosystem. *Applied and Microbiological Biotechnology* 39, 254-258.
- Moser, S.A., Savage, D.C., 2001. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Applied and Environmental Microbiology* 67, 3476–3480.
- Mundy, L.M., Sahm, D.F., Gilmoie, M., 2000. Relationships between enterococcal virulence and antimicrobial resistance. *Clinical Microbiology Reviews* 13, 513-522.
- Murray, B.E., 1990. The life and times of the *Enterococcus*. *Clinical Microbiology Reviews*. 3, 46–65.
- Omar, N.B., Castro, A., Lucas, R., Abrioueh, H., Yousif, N.M.K., Franz, C.M.A.P., Holzapfel, W.H., Pérez-Pulido, R., Martínez-Canamero, M., Gálvez, A., 2004. Functional and safety aspects of enterococci isolated from different Spanish foods. *Systematic Applied Microbiology* 27, 118-130.
- Pinto, M.G.V., Franz, M.A.P., Schillinger, U., Holzapfel, W.H., 2006. *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. *International Journal of Food Microbiology* 109, 205-214.
- Routsis, C., Platsouka, E., Paniara, O., Dimitriadou, E., Saroglou, G., Roussos, C., Armaganidis, A., 2000. Enterococcal infections in a Greek intensive care unit: a 5-y study. *Scandinavian Journal of Infectious Diseases* 32, 275– 280.
- Salminen, S., Deighton, M.A., Benno, Y., Gorbach, S.L., 1998. Lactic acid bacteria in health and disease. In: Salminen, S., Von Wright, A. (Eds.), *Lactic acid bacteria: microbiology and functional aspects*. 2<sup>nd</sup> ed., Marcel Dekker Inc. New York, pp. 1-72.

- Sarem-Damerdj, L.-O., Sarem, F., Marcjal, L., Nicolas, J.-P., 1995. *In vitro* colonization ability of human colon mucosa by exogenous *Lactobacillus* strains. FEMS Microbiology Letters 131, 133-137.
- Satake, S., Clark, N., Rimland, D., Nolte, F., Tenover, F., 1997. Detection of vancomycin resistant enterococci in faecal samples by PCR. Journal of Clinical Microbiology 35, 2325–2330.
- Schmidt, E.J., Boswell, J.S., Walsh, J.P., Schellenberg, M.M., Winter, T.W., Li, C., Allman, C.W., Savage, P.V. 2001. Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria, Journal of Antimicrobial Chemotherapy 47, 671–674.
- Semedo, T., Santos, M.A., Lopes, M.F.S., Marques, J.J.F., Crespo, M.T.B., Tenreiro, R., 2003. Virulence factors in food, clinical and reference enterococci: A common trait in the genus? Systemic Applied Microbiology 26, 13–22.
- Siitonen, S., Vapaatalo, H., Salminen, S., Gordin, A., Saxelin, M., Wikberg, R., Kirkkola, A.-L., 1990. Effect of *Lactobacillus* GG in yoghurt prevention of antibiotic associated diarrhea. Annals of Medicine 22, 57-59.
- Spanhaak, S., Havenaar, R., Schaafsma, G., 1998. The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. European Journal of Clinical Nutrition 52, 899–907.
- Svensson, U., 1999. Industrial perspectives. In: Probiotics: A Critical Review. Tannock, G.W., (Ed.), Horizontal Scientific Press, Wymondham, pp. 57-64.
- Tannock, G.W., Dashkevitz, M.P., Feighner, S.D., 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. Applied and Environmental Microbiology 55, 1848–1851.
- Temmerman, R., Pot, B., Huys, G., Swings, J., 2002. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. International Journal of Food Microbiology 81, 1 – 10.
- Timmerman, H.M., Koning, C.J.M., Muder, L., Rombouts, F.M., Beynen, A.N., 2004. Monostrain, multistain and multispecies probiotic- A comparison of functionality and efficacy: Review article. International Journal of Food Microbiology 96, 219-233.
- Todorov S.D., Wachsman M.B., Knoetze H., Meincken M., Dicks L.M.T., 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soy beans. International Journal of Antimicrobial Agents. 25, 508-513.

- Uhlman, L., Schillinger, U., Rupnow, J.R., Hozhpfel, W.H., 1992. Identification and characterization of two bacteriocin-producing strains of *Lactococcus lactis* isolated from vegetables. International Journal of Food Microbiology 16, 141-151.
- Van den Driessche, M., Peeters, P., Marien, P., Ghooos, Y., Devlieger, H., Veereman-Wauters, G., 1999. Gastric emptying in formula-fed and breast-fed infants measure with the 13C-octanoic acid breath test. Journal of Pediatric Gastroenterology and Nutrition 29 , 46-51.
- Van Reenen, C.A., Dicks, L.M.T., Chikindas, M.L., 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423. Journal of Applied Microbiology 84, 1131-1137.

Table 1  
Residence time of substrates N1 and L1 in each vessel

Vessel	Working volume (ml)	Residence time (h)
1. Stomach	400	2.5
2. Duodenum	600	2.0
3. Jejunum	600	2.0
4. Ileum	600	2.0

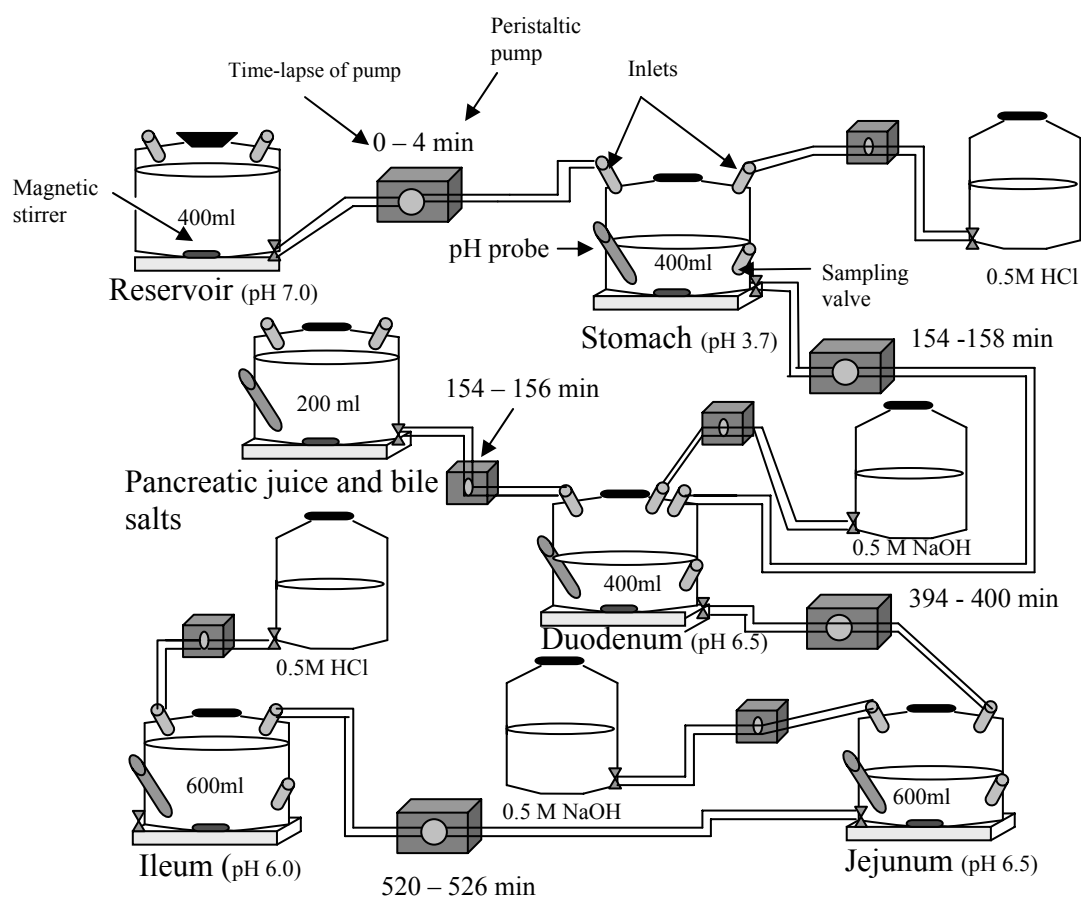


Fig. 1. *In vitro* model simulating the human gastro-intestinal tract

Table 2  
Effect of commercially available medicaments on the growth of selected lactic acid bacteria

Commercial name	Active substance	Concentration (mg/ml)	<i>E. mundtii</i> ST4SA	<i>L. plantarum</i> 423	<i>L. salivarius</i> 241	<i>E. faecium</i> T8	<i>L. johnsonii</i> La1	<i>L. casei</i> Shirota	<i>L. rhamnosus</i> GG
<b>Antibiotics</b>									
Ciprofloxacin	Ciprofloxacin	100	+++	-	+++	+++	++	++	+++
Promoxil	Amoxicillin	100	++++	++++	++++	++++	++++	++++	++++
Cipadur	Cefadroxil	50	+++	+++	+++	+++	++	+++	+++
Roxibid	Roxithromycin	30	+++	+++	+	+	+++	+++	++
Doximal	Doxycycline	20	+++	+++	++++	++++	++++	++++	+++
Utin	Norfloxacin	80	-	-	++	++	-	-	-
<b>Anti-inflammatory medicaments</b>									
Analgin	Metamizole	100	-	-	-	-	-	-	-
Codterpin	Codeine terprim hydrate	50	-	-	-	-	-	-	-
	aminobenzoic acid								
Coxflam	Meloxicam	1.5	++	++	++++	+	++	++	++
Dolocyl	Ibuprofen	40	++	+	-	+	-	-	++
Adco-Ibuprofen	Ibuprofen	40	++	+	-	-	-	+	++
K-fenak	Diclofenac	5	-	-	-	-	-	-	-
Cataflam	Na <sup>+</sup> -diklofenak	5	++	+	+	+	+	+	+
Mefenacid	Na-hydrogen carbonate	100	-	-	-	-	-	-	-
Preflam (Prednisolone)	Benzoic acid, alcohol	3	-	-	-	-	-	+	+++
Rheogesic	Piroxicam	4	+	-	+++	-	-	-	-
<b>Painkillers</b>									
Disprin	Aspirin	60	+	-	-	-	-	-	-
Paracetamol	Paracetamol	100	-	-	-	-	-	-	-
	2-(2,3-dimethylphenyl)								
Pynmed	5 ml contains: paracetamol (120 mg), codeine phosphate (5 mg), promethazine HCl (6.5 mg), alcohol (12.5%, v/v)		++	+	+	+	-	+	+

- = no growth inhibition, + = inhibition zones of 1 - 11 mm in diameter, ++ = inhibition zones of 12 - 16 mm in diameter, +++ = inhibition zones of 17 – 20 mm in diameter, ++++ = inhibition zones larger than 21mm.

Table 3

Primers used in the amplification of certain virulence genes

Virulence property	References	Primer set	Presence of virulence genes
Aggregation substance (AS)	Omar et al., 2004	f: AAG AAA AAG AAG TAG ACC AAC r: AAA CGG CAA GAC AAG TAA ATA	<i>E. mundtii</i> ST4SA
Adhesin to collagen (Ace)	Omar et al. (2004)	f: GAA TTG AGC AAA AGT TCA ATC r: GTC TGT CTT TTC ACT TGT TTC	-
<i>Enterococcus</i> surface protein (Esp)	Omar et al. (2004)	f: TTG CTA ATG CTA GTC CAC GAC r: GCG TCA ACA CTT GCA TTG CCG	<i>E. faecium</i> T8, <i>E. faecium</i> ST 311 LD
<i>Enterococcus faecalis</i> endocarditis antigen (EfaAfs)	Omar et al. (2004)	f: GAC AGA CCC TCA CGA ATA r: AGT TCA TCA TGC TGT AGT	<i>E. faecium</i> T8, <i>E. faecium</i> ST 311 LD
Cytolysin (Cyl)	Omar et al. (2004)	f: TGG GTT GGT GGC GGT ATT TT r: TAA TGC ACC TAC TCC TAA GCC	<i>E. mundtii</i> ST4SA, <i>E. faecium</i> T8, <i>E. faecium</i> ST 311 LD
Non-cytolysin beta $\beta$ hemolysin (hemolysin)	Unpublished	-	<i>E. mundtii</i> ST4SA, <i>E. faecium</i> T8, <i>E. faecium</i> ST 311 LD
Vacomycin (vanA)	Lemcke & Bülte, (2000)	f: TCT GCA ATA GAG ATA GCC GC r: TCT GCA ATA GAG ATA GCC GC	-
Vancomycin (vanB)	Lemcke & Bülte (2000)	f: GCT CCG CAG CCT GCA TGG ACA r: ACG ATG CCG CCA TCC TCC TGC	-
Vancomycin (vanC1)	Clark et al. (1998)	f: GAA AGA CAA AGG AGG ACC GC r: TCG CAT CAC AAG CAC CAA TC	-
Vancomycin (vanC2)	Satake et al. (1997)	f: CGG GGA AGA TGG CAG TAT r: CGC AGG GAC GGT ATT TT	-
Vancomycin (vanC3)	Clark et al. (1998)	f: GCC TTT ACT TAT TGT TCC r: GCT TGT TCT TTG ACC TTA	-

f = forward primer, r = reverse primer, - = virulence genes absent.



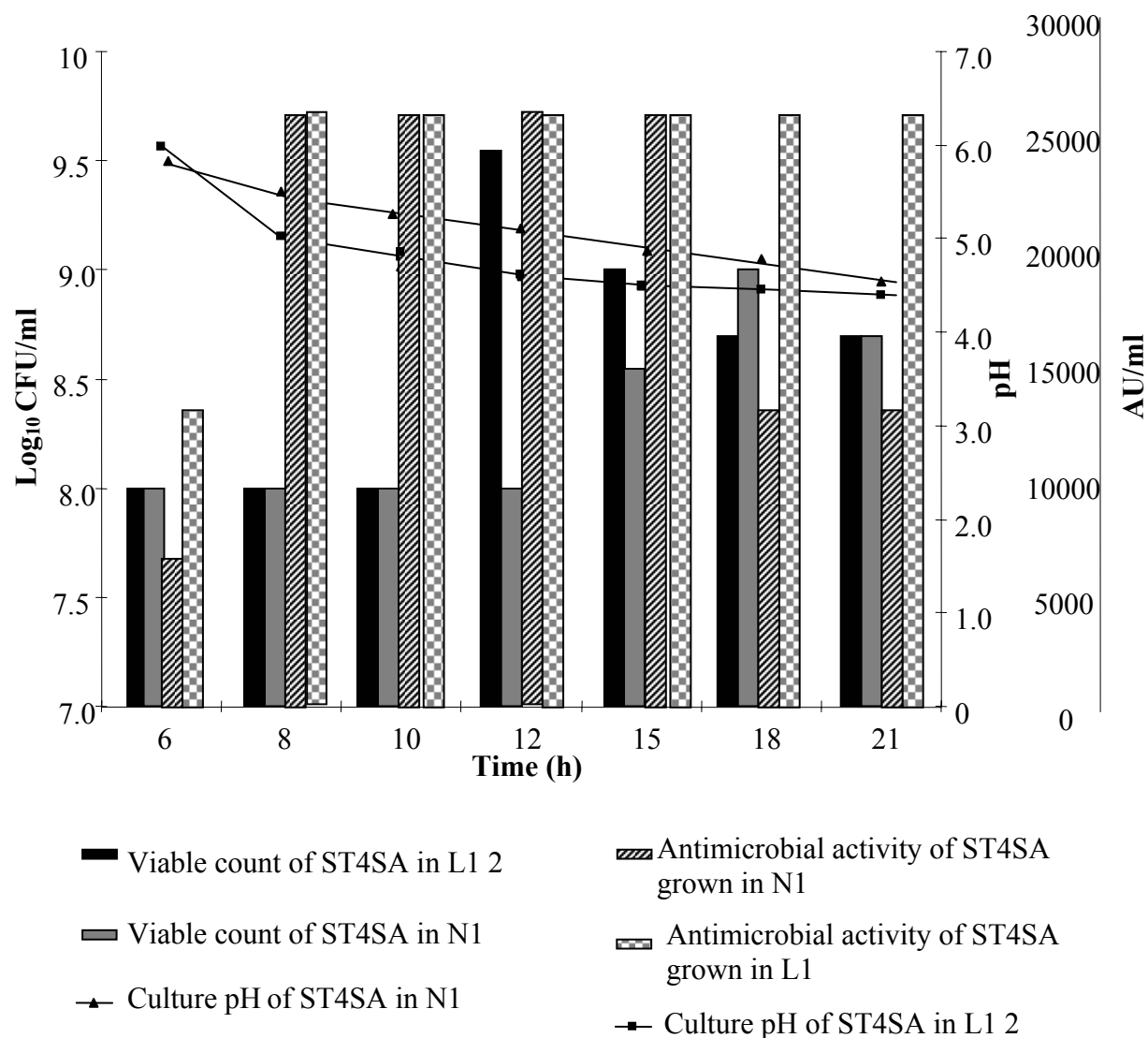


Fig. 2. Viable count of *E. mundtii* ST4SA in N1 and L1 2 for 21 h

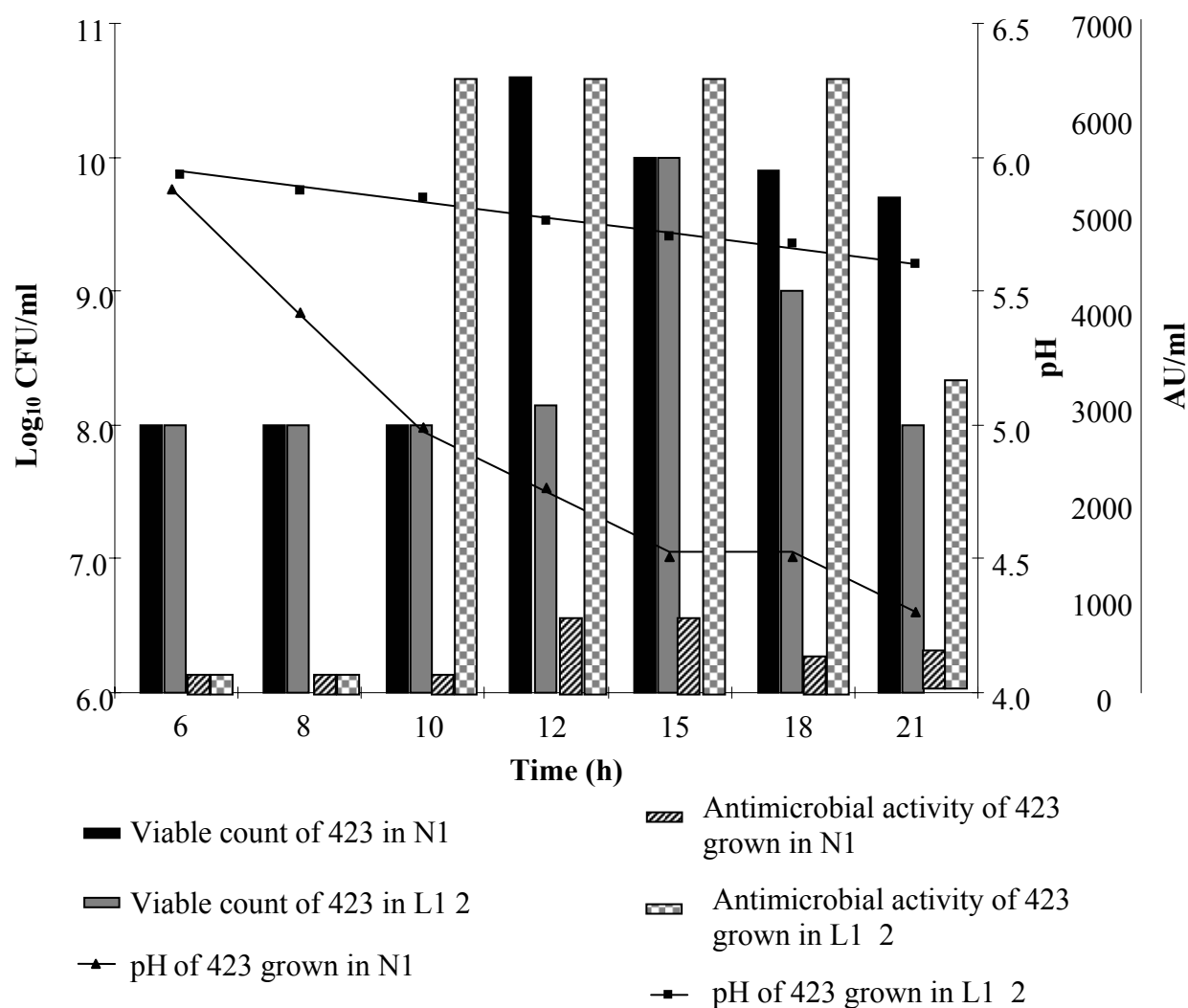


Fig. 3. Viable count of *L. plantarum* 423 in N1 and L1 2 for 21 h

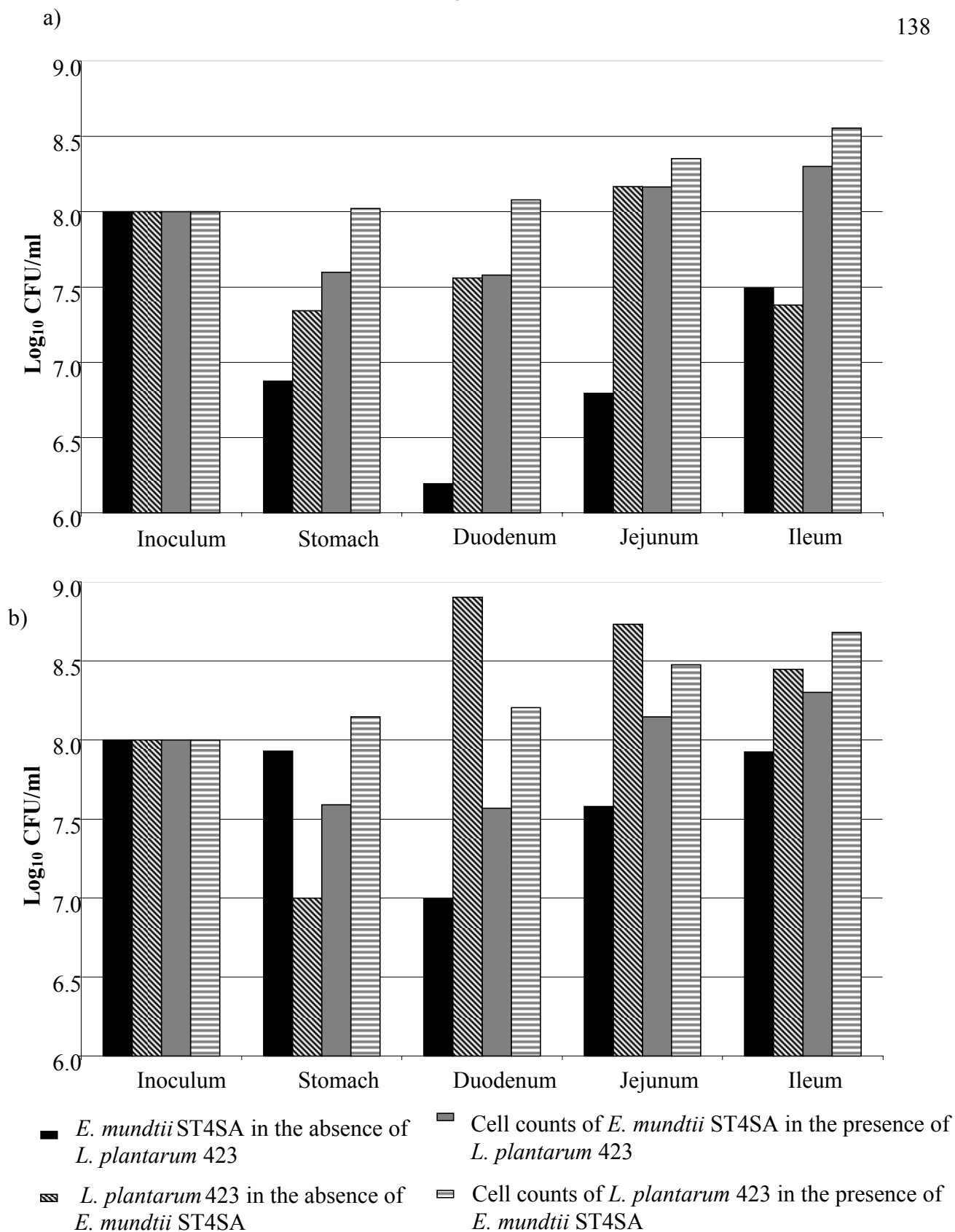


Fig. 4. Survival of *E. mundtii* ST4SA and *L. plantarum* 423 in a) N1 and b) L1 2 in the GIM for 8.5 h

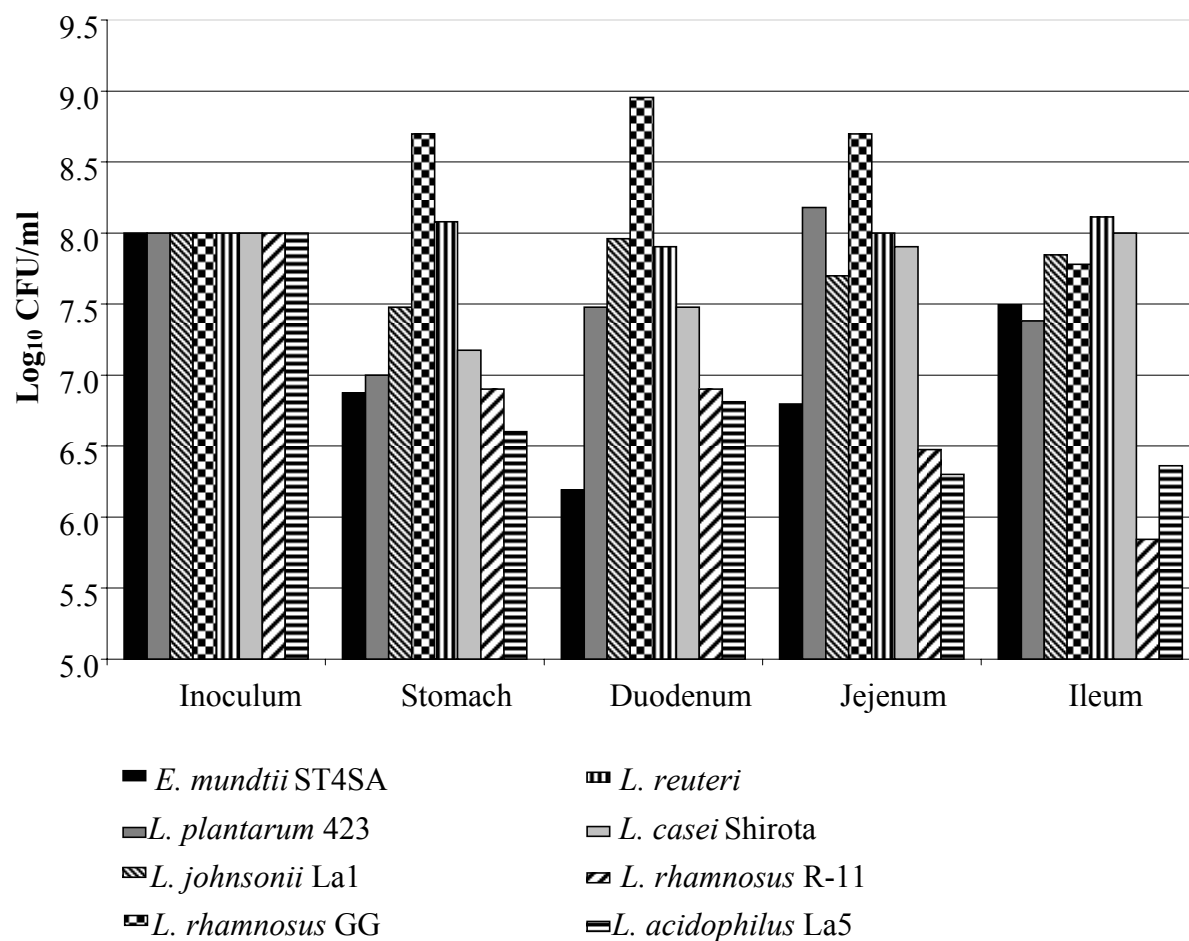


Fig. 5. Survival and growth of *E. mundtii* ST4SA and *L. plantarum* 423, *L. johnsonii* La1, *L. reuteri*, *L. casei* Shirota, *L. rhamnosus* R-11 and *L. acidophilus* La-5 for 8.5 h in the GIM with N1 as substrate.

# CHAPTER 4

## **Adhesion of *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells**

Prepared for publication in Applied and Environmental Microbiology

## **Adhesion of *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells**

MARELIZE BOTES<sup>1</sup>, BEN LOOS<sup>2</sup>, CAROL A. VAN REENEN<sup>1</sup> AND LEON M.T. DICKS<sup>1</sup>.

*Department of Microbiology<sup>1</sup> and Department of Physiological Sciences<sup>2</sup>, University of Stellenbosch, Stellenbosch, 7600, South Africa.*

**Adhesion of *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 (human carcinoma epithelial) cells was visualized by fluorescent staining. Both strains showed good adhesion compared to *Lactobacillus casei* MB1, *L. casei* Shirota, *Lactobacillus johnsonii* La1 and *Lactobacillus rhamnosus* GG. No correlation was found between hydrophobicity, aggregation and adhesion to Caco-2 cells. Presence of antibiotics and anti-inflammatory medicaments reduced adhesion of bacterial strains to Caco-2 cells. Proteins sensitive to pepsin, trypsin and pronase are involved in the adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells. Adhesion of *Listeria monocytogenes* ScottA to Caco-2 cells was not prevented by ST4SA and 423. Cell-free culture supernatants of strains ST4SA and 423 prevented the invasion of *L. monocytogenes* ScottA into Caco-2 cells.**

One of the prerequisites of a good probiotic is adhesion to mucus and epithelial cells (45). Adhesion to the mucosa is, however, influenced by the flow rate, competition for nutrients, adhesion sites and specific physico-chemical properties of the probiotic (20, 48, 55). Adhesion to epithelial cells is facilitated by cell surface carbohydrates, proteins (including S-layer proteins) hemagglutinins and lipoteichoic acids (2, 4, 6, 19, 25, 26, 42, 44, 54).

A number of *in vitro* models have been used to study the adhesion of probiotic cells to epithelial cells. Of these, the colon adenocarcinoma cells Caco-2 and HT-29 are the most popular choice (24, 29, 38, 43, 51). Adherence is strain-specific and is not defined to specific levels in any species. *Lactobacillus rhamnosus* GG, one of the best studied probiotics, adheres at a relatively high level (9.7 %) to Caco-2 cells (17, 51). Strains of *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus acidophilus* and dairy strains of *Lactobacillus rhamnosus*, on the other hand, adhered to Caco-2 cells at levels ranging from 3 to 14 % (5, 8, 18, 24, 39, 48). In general, much higher levels of adhesion (20 to 40 %) have been recorded to mucus producing HT29 MTX cells (48).

Cell lines have also been used to study microbial interactions (1, 47, 56), e.g. endocytosis of *L. monocytogenes* (11, 14, 22, 23, 28, 31, 52). Adhesion of bacteria to epithelial cells is usually studied by subtracting the number of cells that did not adhere to the epithelial cells from the original number of cells before adhesion, by visualizing the cells with Gram-staining (51) or Giemsa staining (18), or by radioactive labeling of the bacteria (12). More recently, fluorescent dyes with specific binding to nucleic acids have been used (21).

In this study, the adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells and the ability to compete with *L. monocytogenes* ScottA were studied. Fluorescent dyes which bind specific to DNA were used. Adhesion of the two strains has been compared to the adhesion of commercially available probiotics. The effect of antibiotics, medicaments, bile, pancreatin, proteolytic enzymes and periodate on adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 were determined.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Lactic acid bacteria (LAB) listed in Table 1 were cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, SA) at 37°C for 18 h. *Listeria monocytogenes* ScottA, used as target strain in competitive exclusion assays, was cultured in Brain Heart Infusion (BHI) (Biolab) at 37°C for 18 h. *Escherichia coli* ATCC 25922 and *Salmonella* sp., used in auto-aggregation and co-aggregation assays, were cultured in BHI at 37°C for 18 h.

**Adhesion of bacterial strains to Caco-2 cells.** Caco-2 cells (Highveld Biological PTY LTD, Kelvin, Johannesburg, SA) were grown in Minimal Essential Medium (MEM) Earle's Base (Highveld Biological), supplemented with 10 % (v/v) fetal bovine serum (FBS) (Sigma, St. Louis, USA), 100 U ml<sup>-1</sup> penicillin (Sigma) and 100 U ml<sup>-1</sup> streptomycin (Sigma). Incubation was at 37°C in the presence of 5 % CO<sub>2</sub>. The media was changed every second day. Adhesion assays were performed with cells at late post-confluence (15 d in culture). Caco-2 cells were seeded at 1 x 10<sup>5</sup> cells per well in 12-well microtitre plates (Multiwell™, Becton Dickinson Labware, NJ, USA) to obtain confluence. Incubation was at 37°C in the presence of 5 % CO<sub>2</sub>. Before the adherence assay, Caco-2 cells were washed twice with sterile phosphate-buffered saline (PBS, 6.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.14 M NaCl, 3.0 mM KCl, pH 7.3).

Cultures of strains listed in Table 1 were harvested (10 000 g, 10 min, 4°C), the cells washed twice with sterile PBS, and diluted in MEM (without FBS, penicillin and streptomycin) to  $OD_{600} = 0.5$  i.e. approximately  $1.0 \times 10^6$  CFU ml<sup>-1</sup>. Wells with Caco-2 cells were inoculated with  $1 \times 10^5$  viable cells of each bacterial cell suspension and incubated at 37°C for 2 h. Non-adhering bacterial cells were then withdrawn from the wells and the Caco-2 cells washed twice with 1 ml sterile PBS, followed by 1 ml 0.5% (v/v) Triton X-100 (Sigma). Bacterial cells were plated onto MRS agar (Biolab) and Listeria Enrichment agar (LEB; Merck, Darmstadt, Germany), respectively. The experiment was conducted in triplicate and the average viable cell numbers determined. The percentage adherence was calculated as described by Shillinger et al. (48):

% Adhesion =  $(cfu/ml_{120} / cfu/ml_0) \times 100$ , where  $cfu/ml_0$  refers to the initial number of viable cells and  $cfu/ml_{120}$  to adhesion of viable bacterial cells after 2 h.

**Determination of cell surface hydrophobicity.** LAB listed in Table 1 were cultured in MRS broth (Biolab) at 37°C for 18 h, harvested (10 000 g, 10 min, 4°C) and washed twice with quarter-strength Ringer's solution (1.5 g NaCl, 0.02 g KCl, 0.03 g CaCl<sub>2</sub> and 0.03 g NaHCO<sub>3</sub>). Optical density readings were recorded at 580 nm (reading 1). To each cell suspension an equal volume n-hexadecane was added and mixed for 2 min. The two phases were allowed to separate for 30 min at 25°C, after which 1 ml of the top phase was removed and the optical density determined (reading 2). The optical density reading was recorded and used to calculate the percentage hydrophobicity according to the equation used by Doyle and Rosenberg (16):

% Hydrophobicity =  $[(OD_{580nm} \text{ reading 1} - OD_{580nm} \text{ reading 2}) / OD_{580nm} \text{ reading 1}] \times 100$ . The experiment was repeated and the average hydrophobicity of each strain calculated.

**Auto-aggregation and co-aggregation.** Eighteen-hour-old cultures of bacteria (Table 1), were harvested (10 000 g, 10 min, 18°C) and washed with sterile saline (0.75 %, w/v, NaCl). The cells were resuspended in saline, adjusted to  $OD_{660} = 0.3$ , and 1 ml of the suspension transferred to a sterile 2 ml plastic cuvette. The cell suspension was centrifuged at 2000 rpm for 2 min. The optical density ( $OD_{660}$ ) of the supernatant was immediately measured ( $OD_0$ ) and after 60 min ( $OD_{60}$ ).

The percentage auto-aggregation was determined according to Malik et al. (34):

% Autoaggregation =  $[(OD_0 - OD_{60}) / OD_0] \times 100$



To investigate co-aggregation of LAB with potential pathogenic organisms, strains of LAB, *L. monocytogenes* ScottA, *E. coli* ATCC 25922 and *Salmonella* sp. (Table 1), were inoculated into the respective growth media and grown to mid-log phase. The cells were harvested (10 000 g, 10min, 18°C) and washed with sterile saline. Equal volumes of cell suspensions of LAB and pathogens were combined. Optical density (OD<sub>660nm</sub>) readings of cells were recorded. The cells were harvested (2000 g, 2 min, 18°C) and the OD<sub>660</sub> of the supernatant determined. The supernatant was discarded. To determine the number of viable cells present in each co-aggregation, serial dilutions of the bottom phase were made, plated onto MRS and BHI agar (Biolab), and the plates incubated at 37°C for 18 h. The percentage co-aggregation was calculated using the equation of Malik et al. (34):

$$\% \text{ Co-aggregation} = [(OD_{\text{Tot}} - OD_s) / OD_{\text{Tot}}] \times 100$$

OD<sub>Tot</sub> = initial OD, taken immediately after the strains were paired and OD<sub>s</sub> = OD of the cell-free supernatant.

**Effect of commercially available medicaments on adhesion of strains.** Caco-2 cells were grown in MEM, supplemented with 10 % (v/v) FBS, 100 U ml<sup>-1</sup> penicillin and 100 U ml<sup>-1</sup> streptomycin. Incubation was at 37°C in the presence of 5 % CO<sub>2</sub>. Caco-2 cells were seeded at 1.0 x 10<sup>5</sup> cells per well in 12-well microtitre plates, as described previously. Before the adherence assay, the Caco-2 cells were washed with sterile PBS. LAB (Table 1) were cultured in MRS (Biolab) to OD<sub>600</sub> = 1.5, harvested (10 000 g, 10 min, 4°C), washed twice with sterile PBS and diluted to OD<sub>600</sub> = 0.5, i.e. equivalent to approximately 1.0 x 10<sup>5</sup> CFU ml<sup>-1</sup> in MEM (without FBS, penicillin and streptomycin). One capsule or tablet of each medicament (Table 3) was diluted in 5 ml sterile water. Tubes with 1 ml MEM (without FBS, penicillin and streptomycin), each supplemented with 100 µl of the eight medicament suspensions, were inoculated into separate microtitre wells, together with each LAB listed in Table 1. In a subsequent study, the concentrations of Cefasyn and Utin were increased 5-fold. Non-adhering bacteria were withdrawn from the wells after 2 h of incubation at 37°C. The Caco-2 cells were washed twice with 1 ml sterile PBS, and then lysed with 1 ml 0.5% (v/v) Triton X-100. Appropriate dilutions were made of the released cells and plated onto MRS agar (Biolab) and incubated at 37°C for 18 h. Controls were adhesion of bacterial strains, inoculated in MEM, without supplementation of antibiotics or medicaments.

**The effect of proteolytic enzymes, ox-bile and pancreatin on adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells.** *E. mundtii* ST4SA and *L. plantarum* 423 were cultured in MRS broth (Biolab) at 37°C for 18 h, washed in 0.8 ml sterile PBS and resuspended in 500 µl of one of the following solutions: 0.1 mol l<sup>-1</sup> citrate-phosphate-0.1 mol l<sup>-1</sup> NaCl, pH 4.5 (buffer A), 0.05 mol l<sup>-1</sup> sodium periodate in buffer A, 0.05 mol l<sup>-1</sup> Tris-HCl-0.1 mol l<sup>-1</sup> NaCl, pH 8.0 (buffer B), 5.0 mg ml<sup>-1</sup> trypsin (Roche Diagnostics GmbH, Mannheim, Germany) in buffer B, 5.0 mg ml<sup>-1</sup> pronase (Roche) in PBS and 5 mg ml<sup>-1</sup> pepsin (Roche) in PBS. The cells were incubated at 37 °C for 1 h, centrifuged (14 000 g, 10 min, 4°C) washed twice in 0.8 ml PBS and resuspended in 1.0 ml of PBS prior to adhesion assays.

Adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells was repeated in the presence of ox-bile (Oxoid Ltd, Hampshire, England) and pancreatin (Sigma) 0.5 % (v/v), 1.0 % (v/v), 1.5 % (v/v) and 2.0 % (v/v), respectively, for 2 h. Adhesion was determined by plate counts as described before. Controls were adhesion of untreated *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells.

**Competitive exclusion of *L. monocytogenes* ScottA.** Competition for adherence of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells was studied by inoculating each well (1.0 x 10<sup>5</sup> Caco-2 cells) with 100 µl (approximately 1.0 x 10<sup>5</sup> cfu) of each strain and 100 µl (approximately 1.0 x 10<sup>5</sup> cfu) of *L. monocytogenes* ScottA. After 2 h of incubation at 37°C, non-adhering bacterial cells were withdrawn from the wells. The Caco-2 cells were washed twice with 1 ml sterile PBS, followed by 1 ml 0.5% (v/v) Triton X-100 and plated onto MRS (Biolab) and LEB agar (Merck).

To determine if strains ST4SA and 423 could prevent adherence of *L. monocytogenes* ScottA to Caco-2 cells, each well (1.0 x 10<sup>5</sup> Caco-2 cells) was inoculated with 100 µl of *E. mundtii* ST4SA or *L. plantarum* 423 (approximately 1.0 x 10<sup>5</sup> cfu) and incubated at 37°C for 1 h. Non-adhering cells were removed by washing Caco-2 cells twice with sterile PBS and then incubated for a further 1 h in the presence of approximately 1.0 x 10<sup>5</sup> cfu *L. monocytogenes* ScottA.

In a separate experiment, the ability of strains ST4SA and 423 to displace *L. monocytogenes* ScottA from Caco-2 cells was determined by first incubating the cells (1.0 x 10<sup>5</sup> per well) in the

presence of *L. monocytogenes* ScottA (approximately  $1.0 \times 10^5$  cfu) at 37°C for 1 h, followed by removal of the bacterial cells as described before, and incubation in the presence of *E. mundtii* ST4SA or *L. plantarum* 423 (approximately  $1.0 \times 10^5$  cfu) for a further 1 h. The wells were washed and the number of viable *E. mundtii* ST4SA and *L. plantarum* 423 released from Caco-2 cells determined by plating onto MRS agar (Biolab) and *L. monocytogenes* ScottA determined by plating onto LEB agar (Merck), respectively.

The competitive exclusion assay was repeated by supplementing MEM (1 ml, without FBS, penicillin and streptomycin) with 5.0 % (v/v), 10 % (v/v), 25 % (v/v), 75 % (v/v) and 100 % (v/v) pH-neutralized cell-free culture supernatants of *E. mundtii* ST4SA and *L. plantarum* 423, respectively. Each well was inoculated with *L. monocytogenes* ScottA (approximately  $1.0 \times 10^5$  cfu). The wells were treated as described before and the number of viable *L. monocytogenes* ScottA released from Caco-2 cells determined by plate counts.

**Cell invasion of *L. monocytogenes* ScottA.** Cell invasion of Caco-2 cells by *L. monocytogenes* ScottA was determined by the aminoglycoside antibiotic assay described by Isberg and Leong (1990). Caco-2 cells were prepared as before and inoculated with 100 µl (approximately  $1.0 \times 10^5$  cfu) *L. monocytogenes* ScottA and incubating at 37°C for 2 h. Non-adhering cells were removed by washing twice with sterile PBS and then incubated for a further 2 h at 37°C in the presence of 40 µg/ml gentamicin (Sigma). The wells were washed twice with PBS, followed by treatment with 1 ml 0.5 % (v/v) Triton X-100 and the number of viable intracellular *L. monocytogenes* ScottA released from the wells determined by plating onto LEB agar (Merck).

To determine the effect of *E. mundtii* ST4SA and *L. plantarum* 423 cell-free culture supernatant on cell invasion of *L. monocytogenes* ScottA, Caco-2 cells were prepared in 12-well microtitre plates as described before. MEM (without FBS, penicillin and streptomycin) was supplemented with 5.0 % (v/v), 10 % (v/v), 25 % (v/v), 75 % (v/v) and 100 % (v/v) pH-neutralized cell-free culture supernatant of both strains, respectively, and inoculated with 100 µl *L. monocytogenes* ScottA (approximately  $1.0 \times 10^5$  cfu). Cell invasion was determined as described before.

**Adhesion of bacterial cells to Caco-2 cells visualized by fluorescent staining.** *E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA were grown to ( $OD_{600nm} = 1.5$ ) and 200

µl of each cell suspension added to 1 ml filter-sterilized water in separate micro-centrifuge tubes. The cells were harvested (10 000 g, 5 min, 18 °C) and the pellet resuspended in 1 ml filter-sterilized water. Three µl hexidium iodide (HI; Molecular probes, Inc., Midrand, SA) from a stock solution (5mg/ml) was added to 1 ml of cell suspension, mixed thoroughly and incubated at 25°C for 15 min in the dark. Caco-2 cells were prepared in eight-chamber slides (Nalge Nunc International, Rochester, NY, USA) as previously described and inoculated with 100 µl MEM (without FBS, penicillin and streptomycin), 0.5 µl Hoechst 33342 (10mg/ml stock solution, Sigma) and stained cells of *E. mundtii* ST4SA and *L. plantarum* 423 (approximately  $1.0 \times 10^4$  viable cells of each strain) in combination and individually. In a separate experiment, Caco-2 cells were inoculated with stained cells of *L. monocytogenes* ScottA (approximately  $1.0 \times 10^3$  viable cells) and in combination with *E. mundtii* ST4SA and *L. plantarum* 423.

Cells were observed on an Olympus cell<sup>^</sup>R system attached to an IX-81 inverted fluorescence microscope equipped with a F-view-II cooled CCD camera (Soft Imaging Systems). Cell<sup>^</sup>R imaging software was used for image acquisition and analysis. For three dimensional imaging, cells were photographed with a step width of 0.26 µm represented in fence view using the cell<sup>^</sup>R software and a 60 x (Olympus PlanApo N) or 100 x (UPlanApo N) oil immersion objective.

For z stack analysis, a defined experiment was set up. Image acquisition parameters such as exposure time, illumination settings and emission filter cube selection were kept constant for all groups and ensured appropriate selection of parameters. The DAPI 360 nm excitation wavelength was used for setting the focal plane, avoiding unnecessary photo-bleaching. For the experiment setting a combined multi color image frame was selected, using 360 nm DAPI for Hoechst, 492 nm and FITC for HI as multiple excitation settings. Through the z-stack frame the top and bottom focus position parameter were selected, indicating the upper and lower dimensions of the cell and a step width of 0.26 µm between the image frames was chosen. For the time-lapse bacterial cells were inoculated to the wells and left to settle for 30 min after which images were taken every five seconds for 20 minutes.

## RESULTS

**Adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells.** Adhesion of strains ST4SA and 423 to Caco-2 cells is expressed as a percentage of the inoculum. Only 6 % cells of *E. mundtii* ST4SA and 8 % cells of *L. plantarum* 423 adhered to Caco-2 cells (Fig. 1). *E. faecium* T8 and *L. rhamnosus* GG adhered slightly stronger at 10.0 % and 9.2 %, respectively. *L. johnsonii* La1, *L. salivarius* 241, *L. casei* MB1 and *L. casei* Shirota adhered at 7.0 %, 6.0 %, 4.5 % and 3.2 %, respectively. *L. monocytogenes* ScottA adhered at 6 % to Caco-2 cells.

**Determination of cell surface hydrophobicity.** No cell surface hydrophobicity was recorded for *E. mundtii* ST4SA (Fig. 2). A hydrophobicity of 50 % was recorded for *L. plantarum* 423, 13 % for *L. casei* Shirota, 28 % for *L. johnsonii* La1, 45 % for *L. casei* MB1 and 54 % for *L. rhamnosus* GG. *E. faecium* T8 and *L. salivarius* 241 revealed the highest hydrophobicity (80 % and 95 %), respectively.

**Auto-aggregation and co-aggregation.** Auto-aggregation ranged from 52 % to 64 % for *E. mundtii* ST4SA, *L. plantarum* 423, *L. salivarius* 241, *E. faecium* T8, *L. casei* Shirota, *L. johnsonii* La1, *L. casei* MB1 and *L. rhamnosus* GG (Fig. 3). Auto-aggregation values between 22 % and 39 % were recorded for *L. monocytogenes* ScottA, *E. coli* ATCC 25922 and *Salmonella* sp. (Fig. 3). The same number cells of *E. mundtii* ST4SA, *L. plantarum* 423, *L. salivarius* 241, *E. faecium* T8, *L. casei* MB1 and *L. rhamnosus* GG cells ( $1.0 \times 10^7$  cfu/ml) co-aggregated with  $1.0 \times 10^7$  cfu/ml *L. monocytogenes* ScottA (Table 2). No cells of *L. casei* Shirota and *L. johnsonii* La1 co-aggregated with *L. monocytogenes* ScottA. Co-aggregation with *E. coli* ATCC 25922 yielded lower cell numbers of *E. mundtii* ST4SA, *L. plantarum* 423, *L. salivarius* 241, *E. faecium* T8, *L. johnsonii* La1 and *L. rhamnosus* GG (Table 2). *L. casei* Shirota and *L. casei* MB1 did not co-aggregate with *E. coli* ATCC 25922. Cells of *E. mundtii* ST4SA, *L. plantarum* 423, *L. salivarius* 241 and *E. faecium* T8 ( $1.0 \times 10^4$  cfu/ml) co-aggregated with  $1.0 \times 10^7$  cfu/ml *Salmonella* sp. Cells of *L. johnsonii* La1 and *L. rhamnosus* GG ( $1.0 \times 10^8$  cfu/ml) co-aggregated with  $1.0 \times 10^7$  cfu/ml *Salmonella* sp. *L. casei* Shirota and *L. casei* MB1 did not co-aggregate with *Salmonella* sp. (Table 2).

**Effect of commercially available medicaments on adhesion to Caco-2 cells.** *E. mundtii* ST4SA, *L. plantarum* 423, *L. salivarius* 241, *E. faecium* T8 *L. casei* MB1, *L. casei* Shirota, *L. johnsonii* La1 and *L. rhamnosus* GG were sensitive to amoxicillin (8 mg/ml), cefadroxil (5 mg/ml), doxycycline (2 mg/ml) and roxithromycin (10 mg/ml) and adhesion to Caco-2 cells decreased in the presence of these antibiotics (Table 3). Diclofenac (0.5 mg/ml) had the least inhibitory effect on the adhesion of *E. mundtii* ST4SA, *L. plantarum* 423, *L. salivarius* 241 and *E. faecium* T8 to Caco-2 cells. However, adhesion of *L. casei* MB1, *L. casei* Shirota, *L. rhamnosus* GG and *L. johnsonii* La1 to Caco-2 cells decreased significantly in the presence of diclofenac (0.5 mg/ml). Norfloxacin (8 mg/ml) had no effect on adhesion of *E. mundtii* ST4SA and *L. salivarius* 241 to Caco-2 cells. Very low adhesion of all the strains was reported in the presence of a five-fold increase in cefuroxime and norfloxacin, except for *L. salivarius* 241 and *L. johnsonii* La1, respectively. Of all strains, *L. salivarius* 241 and *L. johnsonii* La1 showed the highest resistance to antibiotics and anti-inflammatory medicaments in the adhesion assay. *L. rhamnosus* GG was not resistant to any of the antibiotics or anti-inflammatory medicaments (Table 3).

**The effect of proteolytic enzymes, ox-bile and pancreatin on adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells.** Treatment of *E. mundtii* ST4SA and *L. plantarum* 423 with trypsin decreased their adhesion to Caco-2 cells by 3.0 %. Treatment of *E. mundtii* ST4SA with pepsin reduced adhesion by 0.7 %. *L. plantarum* 423 treated with pepsin resulted in a 3.2 % loss in adhesion. Pronase decreased adhesion of strain ST4SA by 4.0 % and strain 423 by 7.5 %, whereas periodate oxidation had no effect on adhesion (Fig. 4). The presence of ox-bile almost completely prevented the adhesion of ST4SA and 423 cells to Caco-2 cells. Pancreatin reduced adhesion by 2.0 to 3.0 %, depending on the concentration (Fig. 4).

**Competitive exclusion of *L. monocytogenes* ScottA.** *L. monocytogenes* ScottA adhered at a rate of 5 % to Caco-2 cells. *E. mundtii* ST4SA did not have an effect on the adhesion of *L. monocytogenes* ScottA, irrespective of the strain being added before, during or after incubation with the pathogen. Adhesion of *L. plantarum* 423 to Caco-2 cells decreased by 1.0 % when *L. monocytogenes* ScottA was added prior to incubation. Cell-free culture supernatant of *E. mundtii* ST4SA and *L. plantarum* 423 had no effect on the adhesion of *L. monocytogenes* ScottA.



**Cell invasion of *L. monocytogenes* ScottA.** Plate counts revealed that from each well an average of  $3 \times 10^1$  cfu/ml viable cells of *L. monocytogenes* ScottA invaded Caco-2 cells. In the presence of 5 % cell-free culture supernatant, both strains did, however, decrease cell invasion of *L. monocytogenes* ScottA by 33%. An increase to 10 % totally inhibited cell invasion.

**Adhesion of bacterial cells to Caco-2 cells visualized by fluorescent staining.** Bacterial cells stained with HI and Hoechst dyes in the absence of Caco-2 cells are shown in Fig. 5. The nuclei of epithelial cells were brightly stained with HI and Hoechst against a uniformly dark background (Figs. 6 - 8). *E. mundtii* ST4SA stained with Hoechst and HI appeared as blue and green cocci in pairs or short chains. Cells of *L. plantarum* 423 stained with HI and Hoechst were single or arranged in chains or, clusters. *L. monocytogenes* ScottA was observed as small and single blue and green rods. Cells of *E. mundtii* ST4SA and *L. plantarum* 423 adhered to Caco-2 cells within 10 min and increased in numbers after an additional 10 min (Fig. 6). *L. monocytogenes* ScottA adhered 30 min later than *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells (not shown). Z-stack and fence view images (Figs 7 and 8) clearly showed how cells of ST4SA and 423 adhered to the edges, surface and centre of epithelial cells. In the absence of strains ST4SA and 423, *L. monocytogenes* ScottA adhered as a cluster of cells to the surface of and in-between Caco-2 cells, which were clearly malformed (Fig. 7 and 8). However, Caco-2 cells appeared normal when strains ST4SA and 423 were present in combination with *L. monocytogenes* ScottA (Fig. 8).

## DISCUSSION

Adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells (6 % and 8 %, respectively) compared well to that recorded for the probiotic *L. rhamnosus* GG (9.2 %). Adhesion of *E. faecium* T8, *L. salivarius* 241, *L. casei* MB1, *L. casei* Shirota and *L. johnsonii* La1 to Caco-2 cells ranged between 3.2 % and 10 % and corresponded to results obtained for other *Lactobacillus* spp. (5, 8, 18, 24, 39, 48, 51). The best adhesion (14 %) to Caco-2 cells was reported for *L. casei* (Fyos®) (51). The ability to adhere to Caco-2 cells is important in the calculation of dosages. If these results are directly comparable with the *in vivo* situation, approximately  $10^{10}$  cfu/ml of each strain should be incorporated in a supplement to ensure the required probiotic dosage of  $1 \times 10^8$  cfu/ml.

The hydrophobicity potential of strains differed considerably. Hydrophobicity plays a key role in first contact between a bacterial cell and mucus or epithelial cells (48, 55). No correlation existed between hydrophobicity potential and adhesion to Caco-2 cells. *E. mundtii* ST4SA with no hydrophobicity and *L. plantarum* 423 with 50 % hydrophobicity adhered well to Caco-2 cells. *Lactobacillus salivarius* 241 and *E. faecium* T8, on the other hand, revealed high levels of hydrophobicity and adhesion to Caco-2 cells. This is contradictory to the findings of Wadström et al. (55). *Lactobacillus* strains showing high cell surface hydrophobicity adhered in high numbers to porcine enterocytes (55). However, in other studies (38, 45, 48) no correlation was found between hydrophobicity and adhesion to mucus or epithelial cells. These discrepancies may be ascribed to the differences in methods.

Auto-aggregation and co-aggregation are important in the formation of biofilms to protect the host from colonization by pathogens (3, 10, 35, 37). Both strains (ST4SA and 423) formed strong aggregates which may increase adhesion to epithelial cells. The two strains also showed good co-aggregation with *L. monocytogenes* ScottA, *E. coli* ATCC 25922 and *Salmonella* sp. Co-aggregation with a potential pathogen allows the probiotic strain to produce antimicrobial substances in a very close proximity of them which may inhibit the growth of pathogenic strains in the gastrointestinal and urogenital tracts (41). Similar results were recorded with *E. faecium* T8, *L. salivarius* 241, *L. johnsonii* La1 and *L. rhamnosus* GG. *L. casei* Shirota did not co-aggregate with *L. monocytogenes* ScottA, *E. coli* ATCC 25922 and *Salmonella* sp. and no co-aggregation of *L. casei* MB1 with *E. coli* ATCC 25922 and *Salmonella* sp. was observed.

Prolonged treatment with antibiotics may lead to strain or species selection and cause an imbalance in the intestinal microflora (16). Probiotic bacteria resistant to antibiotics restore the microbial balance and prevent antibiotic side effects (30). *Enterococcus* spp. are known to be resistant to cephalosporins, low levels of amino-glycoside and clindamycin (36, 50). *Lactobacillus*, *Pediococcus* and *Leuconostoc* spp. have a high natural resistance to vancomycin (27, 49). Our results have shown that the effect of antibiotics and anti-inflammatory medicaments on bacterial adhesion is strain specific. Adhesion to Caco-2 cells decreased, especially in the presence of cefuroxime and norfloxacin. To our knowledge, this is the first evidence of adhesion being influenced by antibiotics or anti-inflammatory medicaments and should therefore be carefully selected when used in combination with probiotics.



A significant proportion ST4SA and 423 cells remained attached to Caco-2 cells after extensive washing. This suggests that adhesion may entail more than non-specific physical entrapment of cells. Treatment with trypsin and pepsin decreased the adhesion of both *E. mundtii* ST4SA and *L. plantarum* 423 by 0.7 % and 3.0 % respectively and it may be assumed that the protein adhesins in the cell membranes were not as sensitive to these proteolytic enzymes. The adhesins in the cell membrane of *L. plantarum* 423 appeared more sensitive to pronase than those of *E. mundtii* ST4SA. Treatment with periodate did not affect adhesion, suggesting that carbohydrates do not play a role in adhesion of *E. mundtii* ST4SA and *L. plantarum* 423. Ox-bile had a detrimental effect on the adhesion of both strains, whilst pancreatin decreased adhesion only slightly. Adsorption of bile to the surface of LAB may induce metabolic changes, leading to altered carbohydrate compositions and changes in adhesion properties (57).

*E. mundtii* ST4SA and *L. plantarum* 423 had no affect on the adhesion of *L. monocytogenes* ScottA to Caco-2 cells. The reason for this phenomenon is not known. Strains ST4SA and 423 may bind to receptors on epithelial cells that are not recognised by *L. monocytogenes* ScottA. The supernatants of *E. mundtii* ST4SA and *L. plantarum* 423 did however prevent *L. monocytogenes* ScottA from invading Caco-2 cells. However, the supernatants had no inhibitory effect on *L. monocytogenes* ScottA during the 2 h adhesion assay and may be due to time restriction. In this assay gentamycin was used to kill cells of *L. monocytogenes* ScottA adhering to intestinal brush border and not those invading Caco-2 cells (9). Cell-free supernatants may interfere with the interaction between pathogens and epithelial cells and prevent the cell-entry process (6, 7, 12, 32, 33). One such example is *L. acidophilus* LB, which produced extracellular components that inhibited signal-dependant cell damage induced by *S. enterica* serovar Typhimurium in Caco-2 cells (32).

The first step of listeriosis is an association of *L. monocytogenes* with microvilli on the plasma membrane and entering of the epithelium from the apical side to invade enterocytes (40). Infection of Caco-2 cells with *L. monocytogenes* ATCC 7644 led to 70% apoptosis and 30% cell lysis after 48 h (53). In this study the cell structure and nuclei of Caco-2 cells changed in the presence of *L. monocytogenes* ScottA, which suggested the initiation of invasion. Caco-2 cells appeared normal in the presence of *E. mundtii* ST4SA and *L. plantarum* 423 despite an increase

in bacterial cells attaching to Caco-2 cells. The cell structure of Caco-2 cells remained normal in the presence of *L. monocytogenes* ScottA, *E. mundtii* ST4SA and *L. plantarum* 423. It is suggested that strains ST4SA and 423 inhibited the deformation of Caco-2 cells by preventing the *L. monocytogenes* ScottA from entering the Caco-2 cells.

Caco-2 cells are useful to collect background information on the adhesion and competitive exclusion abilities of potential probiotic strains. The use of fluorescent proteins i.e. GFP and YFP to distinguish between bacterial cells in the adhesion process is recommended in the future.

## ACKNOWLEDGEMENTS

This study was funded by the National Research Foundation (NRF) of South Africa.

## REFERENCES

1. Alam, M., Miyoshi, S., Yamamoto, S., Tomochika, K. and Shinoda, S. 1996. Expression of virulence-related properties by and intestinal adhesiveness of *Vibrio mimicus* strains isolated from aquatic environments. Appl. Environ. Microbiol. **62**:3871-3874.
2. Alderberth, I., Ahrné, S., Johansson, M.-L., Molin, G., Hanson, L.A. and Wold, A.E. 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. Appl. Environ. Microbiol. **62**:2244-2251.
3. An, Y. and Friedman, R. 2000. Mechanism of bacterial adhesion a pathogenesis of implant and tissue infections. In An, Y. and Friedman, R. (ed.), Handbook of bacterial adhesion. Principles, methods and applications, Totowa, New Jersey, Humana Press.
4. Andreu, A., Stapleton, A.E., Fennell, C.L., Hillier, S.L. and W. E. Stamm, W.E. 1995. Hemagglutination, adherence and surface properties of vaginal *Lactobacillus* species. J. Infect. Dis. **171**:1237–1243.
5. Baccigalupi, L., Donato, A.D., Parlato, M., Luongo, D., Carbone, V., Rossi, M., Ricca, M. and De Felice, M. 2005. Small surface-associated factors mediate adhesion of a food-isolate strain of *Lactobacillus fermentum* to Caco-2 cells. Res. Microbiol. **156**:830-836.

6. **Bernet, M.F., Brassart, D., Neeser, J.R. and Servin, A.L.** 1994. *Lactobacillus acidophilus* La1 binds to cultured human intestinal cell lines and inhibits cell-attachment and cell invasion by enterovirulent bacteria. *Gut*. **35**:483-489.
7. **Bernet-Camard, M.F., Lievin, V., Brassart, D., Neeser, J.R., Servin, A.L. and Hudault, S.** 1997. The human *Lactobacillus acidophilus* strain LA1 secretes a nonbacteriocin antibacterial substance(s) active *in vitro* and *in vivo*. *Appl. Environ. Microbiol.* **63**:2747–2753.
8. **Bertazzoni-Minelli, E., Benini, A., Marzotto, M., Sbarbati, A., Ruzzenente, O., Ferrario, R., Hendriks, H. and Dellaglio, F.** 2004. Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional foods. *Int. Dairy Jnl.* **14**:723-736.
9. **Bibiloni, R., Pérez, P.F. and DeAntoni, G.L.** 1999. Will a high adhering capacity in a probiotic strain guarantee exclusion of pathogens from intestinal epithelia? *Anaerobe*. **5**:519-524.
10. **Boris, S., Suarez, J.E. and Barbés, C.** 1997. Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. *J. Appl. Microbiol.* **83**:413.
11. **Bubert, A., Kuhn, M., Goebel, W. and Köhler, S.** 1992. Structural and functional properties of the p60 proteins from different *Listeria* species. *J. Bacteriol.* **174**:8166–8171.
12. **Coconnier, M.H., Lievin, V., Hemery, E. and Servin, A.L.** 1998. Antagonistic activity against *Helicobacter* infection *in vitro* and *in vivo* by the human *Lactobacillus acidophilus* strain LB. *Appl. Environ. Microbiol.* **64**:4573–4580.
13. **Cohen, P.S. and Laux, D.C.** 1995. Bacterial adhesion to and penetration of intestinal mucus *in vitro*. *Meth. Enzym.* **253**:309-314.
14. **Cossart, P., Pizarro-Cerda, J., Lecuit, M.** 2003. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. *Trends Cell Biol.* **13**:23– 31.
15. **Courvalin, P.** 2006. Antibiotic resistance: The pros and cons of probiotics. *Dig. Liver Dis.* **38**: S261-265.
16. **Doyle, R.J. and Rosenberg, M.** 1995. Measurement of microbial adhesion to hydrophobic substrata. *Methods in Enzymology.* **253**:542-550.

17. **Elo, S., Saxelin and Salminen, S.** 1991. Attachment of *Lactobacillus casei* GG to human colon cell line Caco-2: comparison with other dairy strains. *Lett. Appl. Microbiol.* **13**:154-156.
18. **Forestier, C., De Champs, C., Vatoux, C. and Joly, B.** 2001. Probiotic activities of *Lactobacillus casei rhamnosus*: *in vitro* adherence to intestinal cells and antimicrobial properties. *Res. Microbiol.* **152**:167-173.
19. **Frece, J., Kos, B., Svetec, I.K., Zgaga, Z., Mrša, V. and Šušková, J.** 2005. Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* **982**:285-292.
20. **Freter, R.** 1992. Factors affecting the microecology of the gut. *In* R. Fuller (ed.), *Probiotics. The Scientific Basis* pp. 355-376. Chapman and Hall, London.
21. **Fuller, M.E., Streger, S.H., Rothmel, R.K., Mailloux, B.J., Hall, J.A., Onstott, T.C., Fredrickson, J.K., Balkwill, D.L. and DeFlaun, J.A.** 2000. Development of a vital fluorescent staining method for monitoring bacterial transport in subsurface environments. *Appl. Environ. Microbiol.* **66**:4486-4496.
22. **Gaillard, J.-L., Berche, P., Frehel, C., Gouin, E. and Cossart, P.** 1991. Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci. *Cell.* **65**:1127– 1141.
23. **Gaillard, J.-L., Berche, P., Mounier, J., Richard, S. and Sansonetti, P.** 1987. *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect. Immun.* **55**:2822– 2829.
24. **Gopal, P.K., Prasad, J., Smart, J. and Gill, H.S.** 2001. *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. *Int. J. Food Microbiol.* **67**:207-216.
25. **Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A. and Brassart, D.** 1999. Cell-surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Appl. Environ. Microbiol.* **65**:1071-1077.
26. **Greene, J.D. and Klaenhammer, T.R.** 1994. Factors involved in adherence of Lactobacilli to human Caco-2 cells. *Appl. Environ. Microbiol.* **60**:4487-4494.

27. **Hamilton-Miller, J.M.T. and Shah, S.** 1998. Vancomycin susceptibility as an aid to the identification of lactobacilli. *Lett. Appl. Microbiol.* **26**:153–154.
28. **Kathariou, S., Pine, L., George, V., Carlone, G.M. and Holloway, B.P.** 1990. Nonhemolytic *Listeria monocytogenes* mutants that are also noninvasive for mammalian cells in culture: evidence for coordinate regulation of virulence. *Infect. Immun.* **58**:3988–3995.
29. **Kirjavainen, P., Ouwehand, A., Isolauri, E. and Salminen, S.** 1998. The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiol. Lett.* **167**:185–189.
30. **Knudtson, L.M. and Hartman, P.A.** 1993. Antibiotic resistance among enterococcal isolates from environmental and clinical sources. *J. Food Prot.* **56**:489–492.
31. **Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H. and Cossart, P.** 1992. *L. monocytogenes* ScottA-induced actin assembly requires the actA gene product, a surface protein. *Cell.* **68**:521–531.
32. **Lammers, K.M., Helwig, U., Swennen, E., Rizzello, F., Venturi, A., Caramelli, E., Kamm, M.A., Brigidi, P., Gionchetti, P. and Campieri, M.** 2002. Effect of probiotic strains on interleukin 8 production by HT29/19A cells. *Am. J. Gastroenterol.* **97**:1182–1186.
33. **Lehto, E.M. and Salminen, S.J.** 1997. Inhibition of *Salmonella typhimurium* adhesion to Caco-2 cell cultures by *Lactobacillus* strain GG spent culture supernate: only a pH effect? *FEMS Immunol. Med. Microbiol.* **18**:125–132.
34. **Malik, A., Sakamoto, M., Hanazaki, S., Osawa, M., Suzuki, T., Tochigi, M. and Kakii, K.** 2003. Coaggregation among nonflocculating bacteria isolated from activated sludge. *Appl. Environ. Microbiol.* **69**:6056–6063.
35. **Marsh, P.D. and Bradshaw, D.J.** 1995. Dental plaque as biofilm. *J. Indust. Microbiol.* **15**:169.
36. **Mazza, P.** 1994. The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. *Bull. Chim. Farm.* **133**:3–18.
37. **Ocaña, V.S. and Nadar-Macías, M.E.** 2002. Vaginal lactobacilli: self-and co-aggregating ability. *Br. J. Biomed. Sci.* **59**:183–190.

38. **Ouwehand, A.C., Kirjavainen, P.V., Grönland, M.-M., Isolauri, E. and Salminen, S.J.** 1999. Adhesion of probiotic micro-organisms to intestinal mucus. *Int. Dairy Jnl.* **9**:623-630.
39. **Pennacchia, C., Vaughan, E.E. and Villani, F.** 2006. Potential probiotic *Lactobacillus* strains from fermented sausages: Further investigations on their probiotic properties. *Meat Science.* **73**: 1, 90-101.
40. **Rácz, P., Tenner, K. and Mérö, E.** 1972. An electron microscopic study of the epithelial phase in experimental listeria infection. *Lab. Invest.* **26**: 694-700.
41. **Reid, G., McGroarty, J.A., Angotti, R. and Cook, R.L.** 1988. *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability of uropathogens. *Can. Jnl. Microbiol.* **34**:344-351.
42. **Roos, S. and Jonsson, H.** 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiol.* **148**:433-442.
43. **Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stamatii, A. and Zucco, F.** 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxic.* **21**:1-26.
44. **Satoh, E., Leer, R.J., Conway, P.L. and Pouwels, P.H.** 1999. Mucus adhesion promoting protein of *Lacobacillus reuteri* 104R: 6<sup>th</sup> Symposium on Lactic acid Bacteria, Veldhoven, the Netherlands. 9.
45. **Savage, D.C.** 1992. Growth phase, cellular hydrophobicity, and adhesion *in vitro* of lactobacilli colonizing the keratinizing gastric epithelium in the mouse. *Appl. Environ. Microbiol.* **58**:1992-1995.
46. **Savage, D.C.** 1983. Mechanism by which indigenous microorganisms colonize the gastrointestinal epithelial surfaces. *Prog. Food. Nutr. Sci.* **7**:65-74.
47. **Scaletsky, I.C.A., Fabbricotti, S.H., Aranda, K.R., Morais, M.B. and Fagundes-Netos, U.** 2002. Comparison of DNA hybridization and PCR assays for detection of putative pathogenic enteroadherent *Escherichia coli*. *Clin. Microbiol.* **40**:1254-1258.
48. **Schillinger, U., Guigas, C. and Holzhapfel, W.H.** 2005. *In vitro* adherence and other properties of lactobacilli used in probiotic yoghurt-like products. *Int. Diary J.* **15**:1289-1297.

49. **Simpson, W.J., Hammond, J.R.M. and Miller, R.B.** 1988. Avoparcin and vancomycin-  
Useful antibiotics for the isolation of brewery lactic acid bacteria. *J. Appl. Bacteriol.*  
**64**:299– 309.
50. **Teuber, M.** 1999. Spread of antibiotic resistance with food-borne pathogens. *Cell. Mol.*  
*Life Sci.* **56**:755– 763.
51. **Tuomola, E.M. (née Lehto) and Salminen, S.J.** 1998. Adhesion of some probiotic and  
dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int. J. Food Microbiol.* **41**:45-51.
52. **Va'zquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal,**  
**G., Goebel, W., Gonzá'les-Zorn, B., Wehland, J. and Kreft, J.** 2001. *Listeria*  
pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* **14**:584– 640.
53. **Valenti, P., Greco, R., Pitari, G., Rossi, P., Ajelo, M., Melino, G. and Anonini, G.**  
1999. Apoptosis of Caco-2 intestinal cells invaded by *Listeria monocytogenes*: Protective  
effect of Lactoferrin. *Exp. Cell Res.* **250**: 197-202.
54. **Vidal, K.A., Donnet-Hughes and Granato, D.** 2002. Lipoteichoic acids from  
*Lactobacillus johnsonii* strain La1 and *Lactobacillus acidophilus* strain La10 antagonize  
the responsiveness of human intestinal epithelial HT29 cells to lipopolisaccharide and  
gram-negative bacteria. *Infect. Immun.* **70**:2057-2064.
55. **Wadström, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S. and Gullmar,**  
**B.** 1987. Surface properties of lactobacilli isolated from the small intestine of pigs. *Jnl.*  
*Appl. Bacteriol.* **62**:513-520.
56. **Weinstein, D.L., O'Neill, B.L., Hone, D.M., Metcalf, E.S.** 1998. Differential early  
interactions between *Salmonella enterica* serovar *Typhi* and two other pathogenic  
*Salmonella* serovars with intestinal epithelial cells. *Infect. Immun.* **66**:2310-2318.
57. **Zavaglia, A.G., Kociubinski, G., Pe'rez, P., Disalvo, E., De Antoni, G.** 2002. Effect of  
bile on the lipid composition and cell surface properties of bifidobacteria. *Jnl. Appl.*  
*Microbiol.* **93**:794-799.



TABLE 1. Bacterial strains used in adhesion, hydrophobicity, aggregation and antibiotic resistance assays

Strain	Origin
<i>Enterococcus mundtii</i> ST4SA <sup>a</sup>	Soybeans
<i>Lactobacillus plantarum</i> 423 <sup>a</sup>	Sorghum beer
<i>Enterococcus faecium</i> T8 <sup>a</sup>	Faeces of infants
<i>Lactobacillus salivarius</i> 241 <sup>a</sup>	Intestinal tract of pigs
<i>Lactobacillus casei</i> MB1 <sup>b</sup>	Probiotic yoghurt drink
<i>Lactobacillus casei</i> Shirota <sup>c</sup>	Human intestine
<i>Lactobacillus johnsonii</i> La1 <sup>c</sup>	Human intestine
<i>Lactobacillus rhamnosus</i> GG <sup>c</sup>	Human intestine
<i>Listeria monocytogenes</i> ScottA <sup>c</sup>	Human intestine
<i>Escherichia coli</i> ATCC 25922 <sup>a</sup>	Human intestine
<i>Salmonella</i> sp. <sup>d</sup>	Human intestine

<sup>a</sup> Culture collection, Department of Microbiology, University of Stellenbosch<sup>b</sup> Isolated from a probiotic yoghurt drink as *L. casei* defenses<sup>c</sup> Received from W.H. Holzapel (Institute of Hygiene and Toxicology, Karlsruhe, Germany)<sup>d</sup> Obtained from Red Cross Children's Hospital, SATABLE 2. Co-aggregation of LAB with *L. monocytogenes* ScottA, *E. coli* ATCC 25922 and *Salmonella* sp.

Strain	Co-aggregation with <i>L. monocytogenes</i> ScottA (%)	Co-aggregation with <i>E. coli</i> ATCC 25922 (%)	Co-aggregation with <i>Salmonella</i> sp. (%)
<i>E. mundtii</i> ST4SA	53.8	45.8	12.7
<i>L. plantarum</i> 423	51.3	43.6	12.9
<i>L. salivarius</i> 241	49.2	49.2	13.3
<i>E. faecium</i> T8	48.8	44.4	12.8
<i>L. casei</i> MB1	42.4	29 <sup>b</sup>	12.2 <sup>c</sup>
<i>L. casei</i> Shirota	41.7 <sup>a</sup>	35.0 <sup>b</sup>	19.9 <sup>c</sup>
<i>L. johnsonii</i> La1	49.2 <sup>a</sup>	53.1	56.8
<i>L. rhamnosus</i> GG	48.3	55.6	60.3

<sup>a</sup> Values obtained for *L. monocytogenes* (no LAB aggregated)<sup>b</sup> Values obtained for *E. coli* ATCC 25922 (no LAB aggregated)<sup>c</sup> Values obtained for *Salmonella* sp. (no LAB aggregated)



TABLE 3. The effect of commercially available antibiotics and anti-inflammatory medicaments on the adhesion of lactic acid bacteria to Caco-2 cells, expressed as percentage values.

Medica- ment	Active substance	Concen- tration mg/ml	<i>E. mundtii</i> ST4SA	<i>L.</i> <i>plantarum</i> 423	<i>E.</i> <i>faecium</i> T8	<i>L.</i> <i>salivarius</i> 241	<i>L. casei</i>	<i>L.</i> <i>casei</i> Shirota	<i>L.</i> <i>rhamnosus</i> GG	<i>L.</i> <i>johnsonii</i> La1
Control	-	-	6.0	8.0	10	6.0	4.5	3.2	9.2	3.3
Cefacyn	Cefuroxime	10	2.4	0.8	1.5	<b>6.0</b>	0	<b>3.0</b>	1.0	<b>3.3</b>
Cefacyn	Cefuroxime	50	0.14	0.004	0.225	<b>6.0</b>	0	0.04	0.05	0.1
Utin	Norfloxacin	8	<b>6.0</b>	0.68	0.45	<b>6.0</b>	<b>4.5</b>	<b>3.2</b>	0.05	<b>3.3</b>
Utin	Norfloxacin	40	0.6	1.2	1.5	2.25	0.1	0.32	0.003	<b>3.3</b>
Cipadur	Cefadroxil	5	1.2	0.32	0.5	1.5	1.0	1.0	0.33	0.7
Roxibudd	Roxithromycin	10	0.6	0.56	0.5	2.25	3.5	2.0	0.05	0.1
Promoxil	Amoxicillin	8	2.5	0.4	3.5	0.36	0.7	2.1	0.33	0.3
Doximal	Doxycycline	2	2.0	0.64	1.0	3.75	0.7	1.2	0.33	1.3
Ibuprofen	Ibuprofen	2	0.16	1.0	1.5	4.5	<b>4.5</b>	<b>3.0</b>	0.83	0
K-fenak	Diclofenac	0.5	<b>6.0</b>	<b>8.0</b>	<b>10</b>	<b>6.0</b>	0.7	1.6	1.0	<b>3.0</b>

Adhesion of bacteria not affected by antibiotics and anti-inflammatory medicaments is printed in bold.

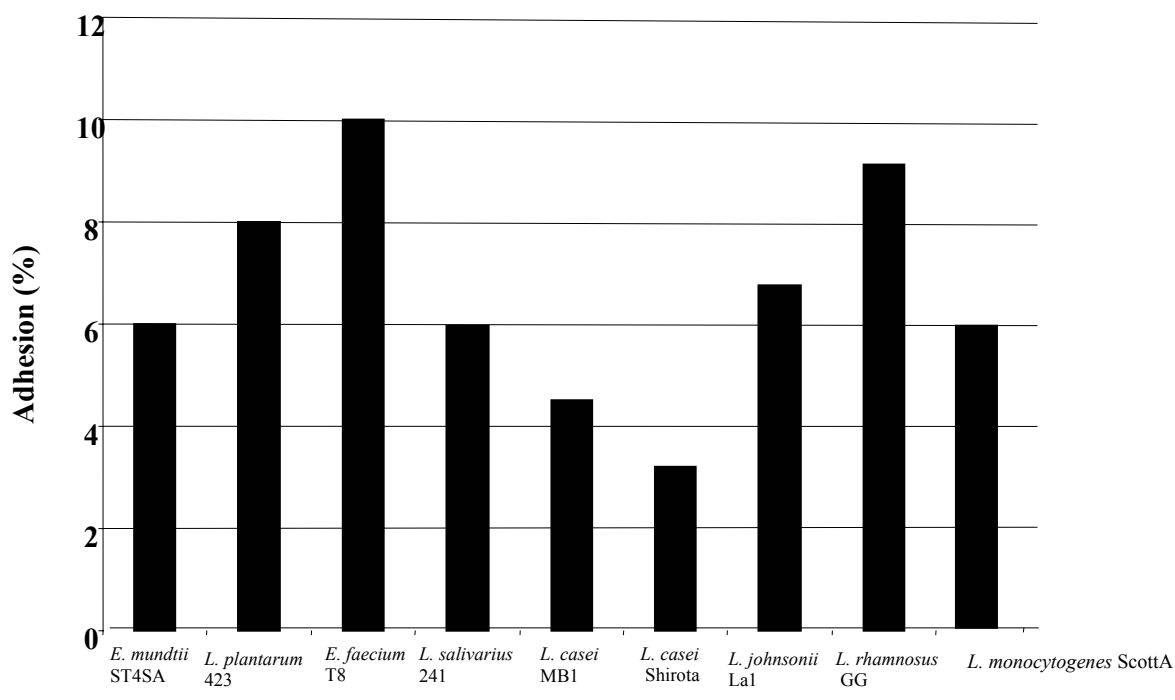


FIG. 1. Adhesion of bacteria to Caco-2 cells.

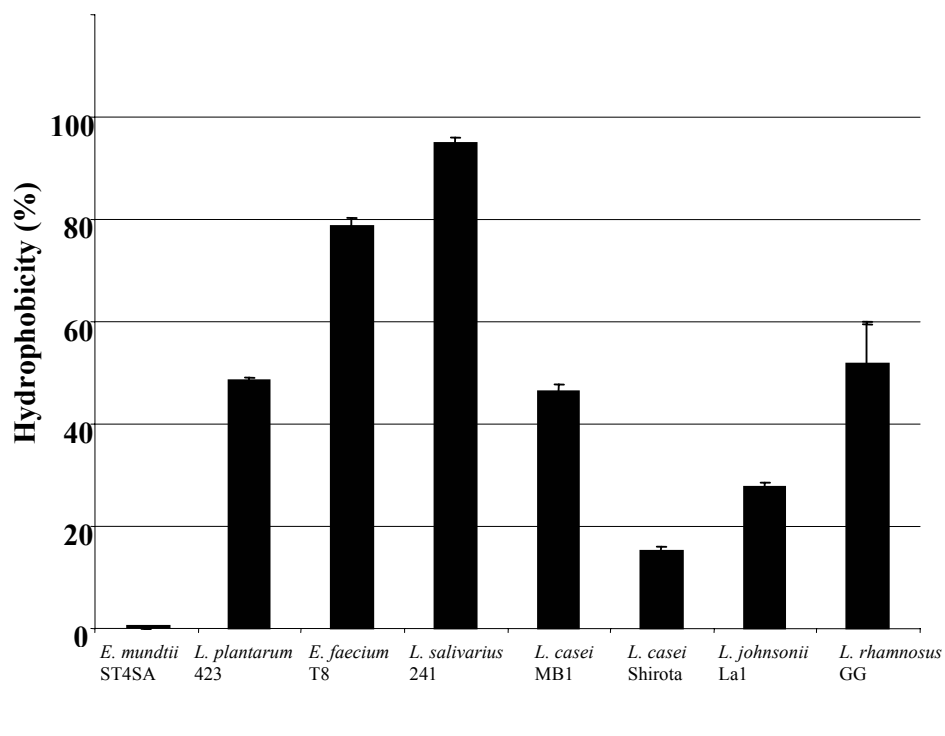


FIG. 2. Hydrophobicity of bacteria, expressed as the mean percentage.

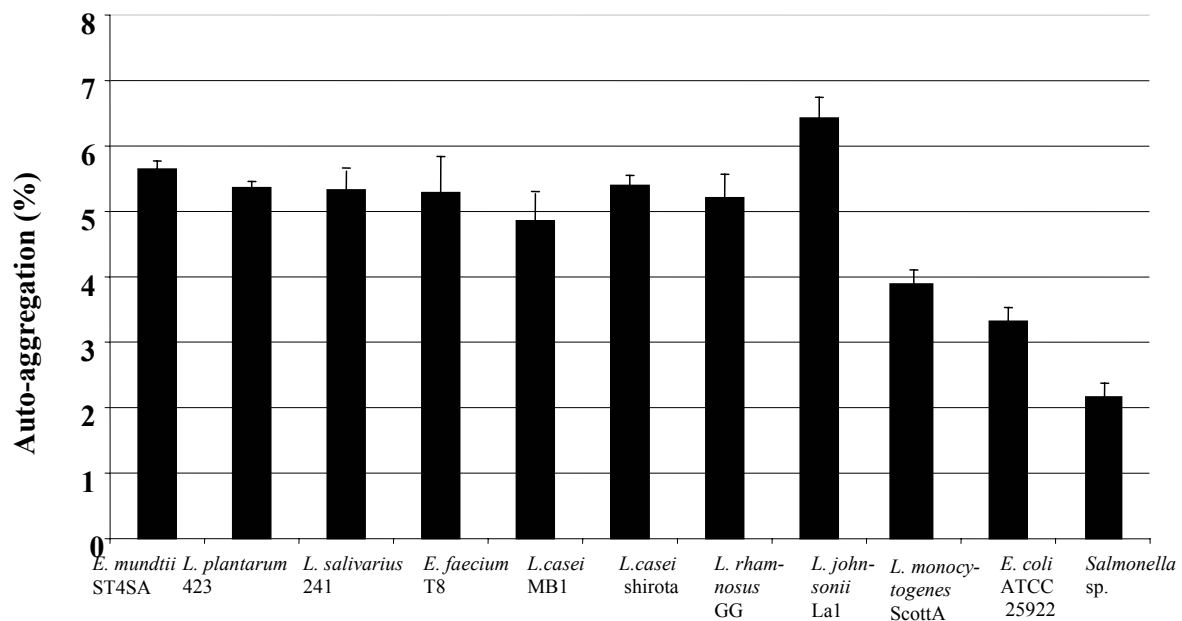


FIG. 3. Auto-aggregation of bacteria expressed as the mean percentage.

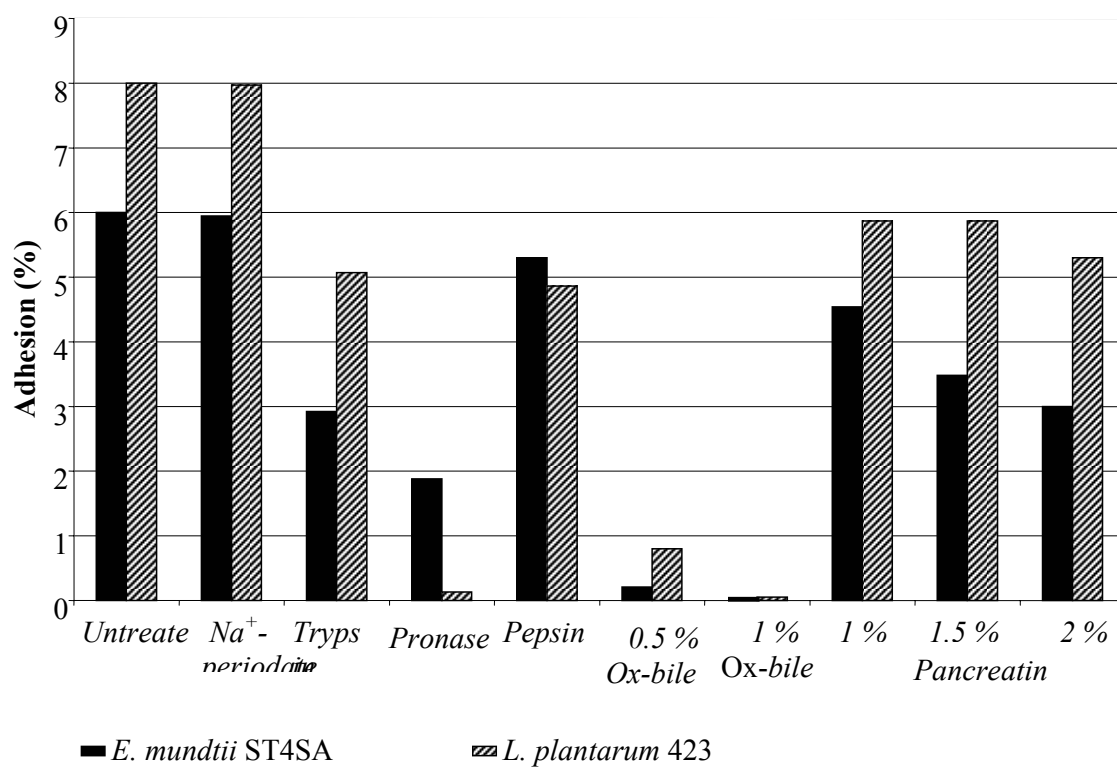


FIG. 4. Adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells after treatment with Na<sup>+</sup>-periodate, trypsin, pronase, pepsin, ox-bile and pancreatin.

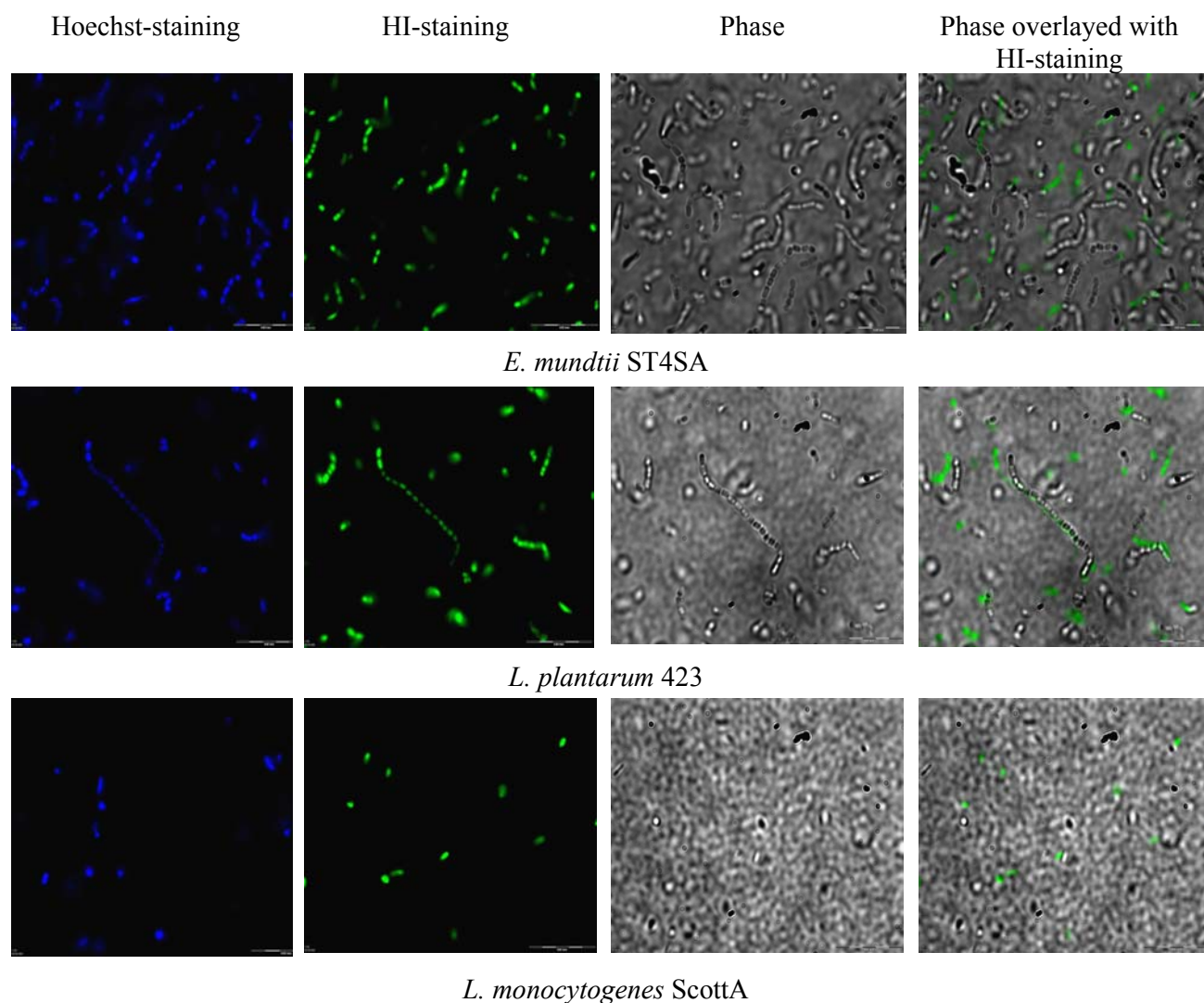


FIG. 5. Images of *E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA stained with HI and Hoechst dyes, in the absence of Caco-2 cells.

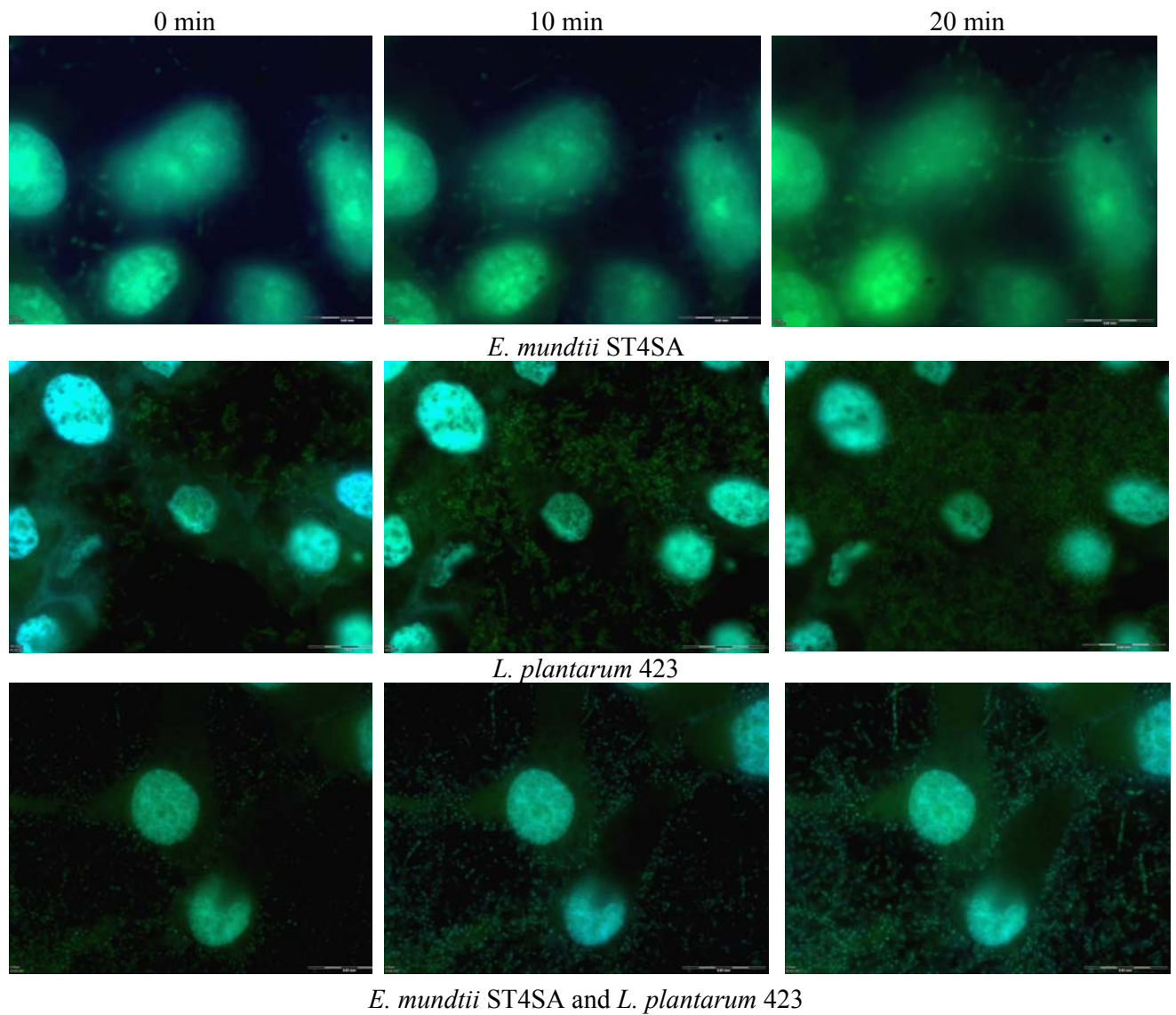


FIG. 6. Adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 to Caco-2 cells, stained with HI and Hoechst, and recorded after 0 min, 10 min and 20 min.



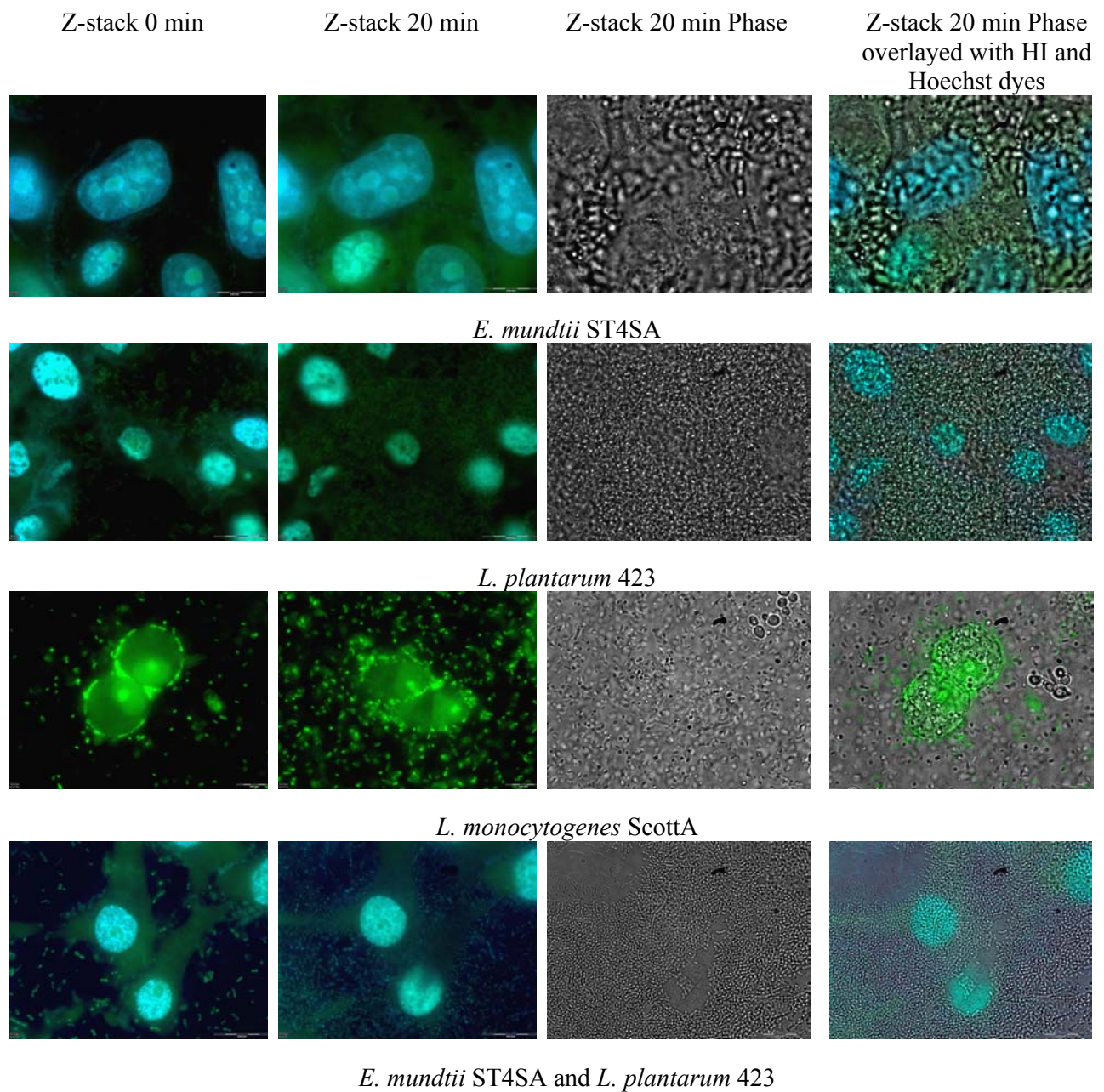
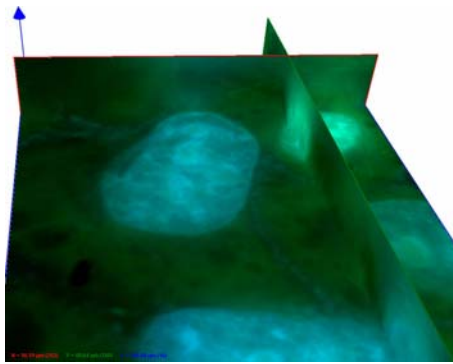
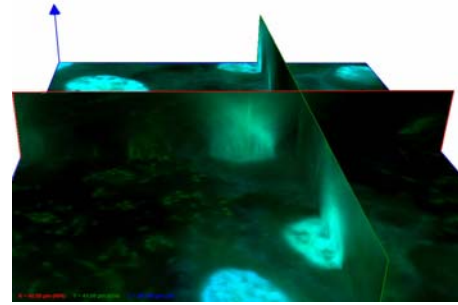


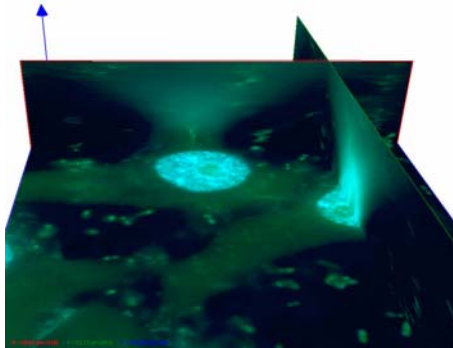
FIG. 7. Z-stack images of *E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA adhering to Caco-2 cells before and after 20 min.



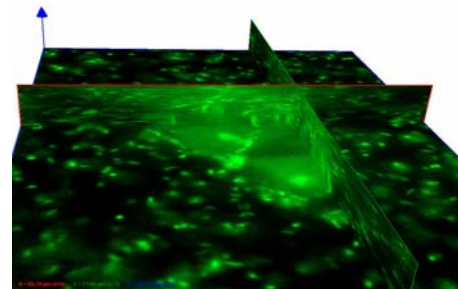
*E. mundtii* ST4SA



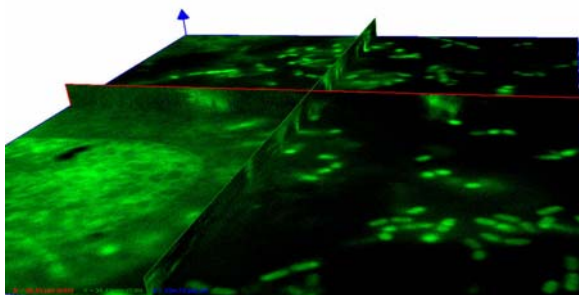
*L. plantarum* 423



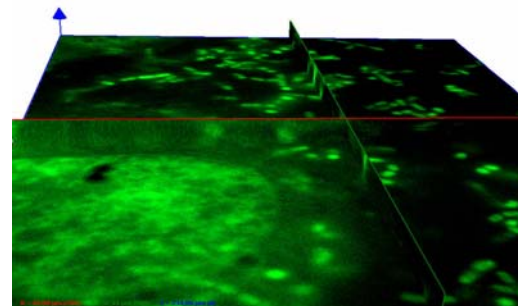
*E. mundtii* ST4SA and *L. plantarum* 423



*L. monocytogenes* ScottA



*E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA a



*E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA b

FIG. 8. Fence view images of *E. mundtii* ST4SA, *L. plantarum* 423 and *L. monocytogenes* ScottA adhering to Caco-2 cells.

# Chapter 5

## **General discussion and conclusions**



## General discussion

The probiotic concept, developed in recent years, involves the selection of lactic acid bacteria for probiotic supplements and functional foods (Gibson & Fuller, 2000). *In vitro* assessment forms part of the initial screening process and may include resistance to gastric and bile acid, adhesion to mucus and/or human epithelial cells, competitive exclusion of pathogenic bacteria and safety (Conway et al., 1987; Floch, 2002; Lin et al., 2006; Saarela et al., 2000). *In vitro* models, simulating the gastrointestinal tract in humans, have been developed to represent *in vivo* conditions (Mainville et al., 2005).

In this study the GIM, simulating the upper gastrointestinal tract of the human infant, was developed. *Enterococcus mundtii* ST4SA (isolated from soy beans) and *Lactobacillus plantarum* 423 (isolated from sorghum beer), active against various Gram-positive and Gram-negative bacteria, were compared to the survival of commercial probiotics. Two infant milk formulas were used as growth media. Strain ST4SA was sensitive to the low pH conditions, bile and pancreatin in the GIM. Cell numbers of *L. plantarum* 423 decreased in low pH conditions and this strain was resistant to bile and pancreatin, despite the absence of bile-salt hydrolase activity. Both strains showed high viability in the ileum component of the GIM and it is considered to increase in cell numbers in the colon. Combination of the two strains improved their survival in the GIM. *L. rhamnosus* GG showed the highest survival of the commercial probiotics tested and strains ST4SA and 423 compared well to this probiotic. Growth of *L. monocytogenes* ScottA in the GIM was inhibited in the presence of strains ST4SA and 423. The production of antimicrobial peptides is the possible explanation for their antimicrobial activity against *L. monocytogenes* ScottA. Previous studies have shown that peptide ST4SA and plantaricin 423 inhibit the growth of *Listeria* spp. (de Kwaadsteniet et al., 2005; Van Reenen et al., 1998).

Although lactic acid bacteria have GRAS status, safety criteria such as antibiotic resistance and inheritance of virulence factors of all potential probiotic strains should be determined. Antibiotic resistance of *E. mundtii* ST4SA and *L. plantarum* 423 correlated to lactobacilli and enterococci in previous studies (Danielsen & Wind, 2003; Landman & Quale, 1997; Leclercq, 1997; Murray, 1990). Strains ST4SA and 423 showed resistance to beta-lactams penicillin, macrolides, tetracycline, quinolones and anti-inflammatory medicaments. *L. plantarum* 423 was the only strain resistant to the quinolone, ciprofloxacin of these tested. Three virulence

factors were found in *E. mundtii* ST4SA, although the genes were not expressed.. No virulence factors were found in *L. plantarum* 423.

Adhesion and colonization of probiotic bacteria in the gastro-intestinal tract of the host is believed to be one of the essential features required for delivering health benefits (Bernet et al., 1994). Caco-2 cells exhibit characteristics of epithelial cells in the small intestine and were used as an adhesion model in this study (Gopal et al., 2001). Adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 compared well to the commercial probiotic strain, *L. rhamnosus* GG. Factors involved in their attachment to Caco-2 cells include auto-aggregation and protein factors sensitive to pepsin, trypsin and pronase, present on the surface of their bacterial cell walls. No carbohydrate factors are involved and hydrophobicity was reported only for strain 423. Bile and antibiotics may change the surface and adhesion properties of bacterial strains and therefore reduced adhesion of strains ST4SA and 423 to Caco-2 cells (Zavaglia et al., 2002). Adhesion of both strains and *L. monocytogenes* ScottA was visualized by fluorescent staining. Cells of strains ST4SA and 423 adhered on the surface and nucleus of the Caco-2 cells and increased in adhesion over time. Cells of *L. monocytogenes* ScottA took longer to adhere to Caco-2 cells than strains ST4SA and 423. A large number of cells attached to the surface and in between the Caco-2 cells.

An important aspect of probiotic bacteria is the protection of the gastro-intestinal micro-environment from invading pathogens (Gopal et al., 2001). The competitive exclusion abilities of *E. mundtii* ST4SA and *L. plantarum* 423 may include co-aggregation and the production of antimicrobial substances. Both strains co-aggregated with *L. monocytogenes* ScottA, *E. coli* ATCC 25922 and *Salmonella* sp. Cells of *L. monocytogenes* ScottA invaded Caco-2 cells and images of Caco-2 cells in the presence of *L. monocytogenes* ScottA confirmed these results. Cell-free culture supernatants of strains ST4SA and 423 did however inhibit the invasion of *L. monocytogenes* ScottA. *E. mundtii* ST4, *L. plantarum* 423 and *L. monocytogenes* ScottA adhering to Caco-2 cells revealed no change in the cell structure of Caco-2 cells. We predict that strains ST4SA and 423 may inhibit the invasiveness and cell association characteristics of *L. monocytogenes* ScottA and prevent Caco-2 cells from listeriosis.

### Concluding remarks and future perspectives

The GIM and Caco-2 cells were useful to predict survival and adhesion of *E. mundtii* ST4SA and *L. plantarum* 423 in the gastro-intestinal tract. *E. mundtii* ST4SA and *L. plantarum* 423 exhibit characteristics generally considered essential for probiotic organisms. Both strains proved to survive GIM transit and will most probably colonize in the ileum and colon.

Strains ST4SA and 423 compared well to the reference strain *L. rhamnosus* GG in terms of survival and adhesion. This probiotic strain is effective in preventing and treating acute diarrhoea in infants (Isolauri et al., 2001). A few studies indicate a relationship between *in vitro* adhesion and *in vivo* colonization (Cesena et al., 2001; Castagliuolo et al., 2005) or modulating the immune system (Schiffrin et al., 1997). Therefore strains ST4SA and 423 may modulate the intestinal immunity in a similar way. *E. mundtii* ST4SA and *L. plantarum* 423 may also be used to prevent gastro-intestinal infection of *L. monocytogenes* ScottA and possibly other pathogens.

Fluorescent staining of bacterial cells was useful in the visualization of adhesion to Caco-2 cells however differentiation between the strains was difficult. Expression of fluorescent reporter genes i.e. *lux* genes in the genome of bacteria may address this problem. *In vivo* fluorescent imaging may identify specific adhesion sites for bacteria in the gastrointestinal tract and the ability to compete with pathogens.

It is recommended that *E. mundtii* ST4SA and *L. plantarum* 423 should be combined when commercialized. Application of both strains as probiotics may include supplementation of infant milk formulations or encapsulated for use as a probiotic supplement. Dosage of each strain should be calculated according to adhesion rates. Strains ST4SA and 423 should not be administered in combination with antibiotics and anti-inflammatory medicaments, with the exception of diclofenak.

However, prior to commercialization, human clinical studies are compulsory to evaluate survival and colonization of *E. mundtii* ST4SA and *L. plantarum* 423 in the gastro-intestinal tract and to identify possible health benefits of each strain.

## References

- Bernet, M.F., Brassart, D., Neeser, J.R., Servin, A.L., 1994. *Lactobacillus acidophilus* La1 binds to cultured human intestinal cell lines and inhibits cell-attachment and cell invasion by enterovirulent bacteria Gut 35, 483-489.
- Cesena, C., Morelli, L., Alander, M., Siljander, T., Tuomola, E., Salminen, S., 2001. *Lactobacillus crispatus* and its nonaggregating mutant in human colonization trials. Journal of Dairy Science 84, 1001-1010.
- Castagliuolo, I., Galeazzi, F., Ferrari, S., Ell, M., Brun, P., Cavaggioni, A. 2005. Beneficial effect of auto-aggregating *Lactobacillus crispatus* on experimentally induced colitis in mice. FEMS Immunology in Medical Microbiology 43, 197-204.
- Conway, P.L., Gorbach, S.L., Goldin, B.R., 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cell. Journal of Dairy Science 70, 1-12.
- Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. International Journal of Food Microbiology 82, 1 –11.
- De Kwaadsteniet, M., Todorov, S.D., Knoetze, H., Dicks, L.M.T., 2005. Characterization of a 3944 kDa bacteriocin produced by *Enterococcus mundtii* ST15, with activity against Gram-positive and Gram-negative bacteria. International Journal of Food Microbiology 105, 433-444.
- Floch, M.H., 2002. Bile salts, intestinal microflora and enterohepatic circulation. Digestive and Liver Disease 34 (Suppl2), S54-57.
- Gibson, R.G., Fuller, R., 2000. Aspects of *in vitro* and *in vivo* research approaches directed toward identifying probiotics and prebiotics for human research. Symposium: Probiotic bacteria: Implications for human health, American Society for Nutritional Sciences, 391S-395S.
- Gopal, P.K., Prasad, J., Smart, J., Gill, H.S., 2001. *In vitro* adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. International Journal in Food Microbiology 67, 207-216.
- Isolauri, E., Sütas, Y., Kankaanpää, Arvilommi, H., Salminen, S., 2001. Probiotics: effects on immunity. American Journal of Clinical Nutrition 73, 444S-450S.
- Landman, D., Quale, J.M., 1997. Management of infections due to resistant enterococci: a review of therapeutic options. Journal of Antimicrobial Chemotherapy 40, 161– 170.
- Leclercq, R., 1997. Enterococci acquire new kinds of resistance. Clinical Infectious Diseases 24 (Suppl.1), S80– S84.

- Lin, W.-H., Hwang, C.-F., Chen, L.-W., Tsen, H.-Y., 2006. Viable counts, characterisatic evaluation for commercial lactic acid bacteria products. Food Microbiology 23, 74-81.
- Mainville, I., Arcand, Y., Farnworth, E.R., 2005. A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. International Journal of Food Microbiology 99, 287-296.
- Murray, B.E., 1990. The life and times of the *Enterococcus*. Clinical Microbiology Reviews 3, 46–65.
- Saarela, M., Mogensen, G., Fondén, R., Mättö, J., Mattila-Sandholm, T., 2000. Probiotic bacteria: safety, functional and technological properties. Journal of Biotechnology 84, 197-215.
- Schiffrin, E.J., Brassard, D., Servin, A.L., Rocha, F., Donnet-Hughes, A., 1997. Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. American Journal of Clinical Nutrition 66, 515S-520S.
- Van Reenen, C.A., Dicks, L.M.T., Chikindas, M.L., 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423. Journal of Applied Microbiology 84, 1131-1137.
- Zavaglia, A.G., Kociubinski, G., Pe´rez, P., Disalvo, E., De Antoni, G., 2002. Effect of bile on the lipid composition and cell surface properties of bifidobacteria. Journal of Applied Microbiology 93, 794-799.